Functional analysis of plant genes in legume nodulation

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### Frequently used abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AM</td>
<td>arbuscular mycorrhiza</td>
</tr>
<tr>
<td>AON</td>
<td>autoregulation of nodulation</td>
</tr>
<tr>
<td>3-AT</td>
<td>3-amino-1,2,4-triazole</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BY-2</td>
<td>Bright Yellow-2</td>
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<tr>
<td>ccd</td>
<td>cortical cell division</td>
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<tr>
<td>CK</td>
<td>cytokinin</td>
</tr>
<tr>
<td>CLE</td>
<td>CLAVATA/ESR-like peptide</td>
</tr>
<tr>
<td>CLV</td>
<td>Clavata genes</td>
</tr>
<tr>
<td>DMI</td>
<td>Doesn't Make Infections</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-inoculation</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>ENOD</td>
<td>early nodulin gene</td>
</tr>
<tr>
<td>EPS</td>
<td>exopolysaccharides</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>GA(s)</td>
<td>gibberellin(s)</td>
</tr>
<tr>
<td>GA20ox</td>
<td>gibberellin-20 oxidase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>IP</td>
<td>infection pocket</td>
</tr>
<tr>
<td>ipt</td>
<td>iso-pentenyltransferase gene</td>
</tr>
<tr>
<td>IT(s)</td>
<td>Infection thread(s)</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>Lb(s)</td>
<td>leghemoglobin(s)</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRB</td>
<td>lateral root base</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich-repeat</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase ($10^6$)</td>
</tr>
<tr>
<td>Myc</td>
<td>mycorrhization</td>
</tr>
</tbody>
</table>
NAC  NAM/CUC/ATAF transcription factor
N₂  atmospheric nitrogen
NF(s)  nod factor(s)
Nod  nodulation
NP  nodule primordium
NSP  nodulation signaling pathway
ORF  open reading frame
qRT-PCR  quantitative reverse transcription polymerase chain reaction
RHC  root hair curling
RLK(s)  receptor-like kinase(s)
RNAi  RNA interference
RNS  root nodule symbiosis
ROS  reactive oxygen species
SAM  shoot apical meristem
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m  standard error measurement
SNF  symbiotic nitrogen fixation
TC(s)  tentative consensus sequence(s)
T-DNA  transfer DNA
wt  wild type
Scope

The symbiotic interaction between legume plants and rhizobia triggers the formation of new root organs, the nodules, in which the bacteria fix atmospheric nitrogen. This mutuality provides the bacteria with a niche and carbon sources and benefits the plant with an unlimited nitrate source, essential for plant growth and development. Legumes are indispensable in a sustainable ecological environment making it even more challenging to study the nodulation process on a plant molecular level to unravel the sequential signaling mechanisms required to fulfill this symbiosis.

In this study, the water-tolerant legume Sesbania rostrata was used as a model to study the plant’s perception and downstream transduction mechanisms after recognition of the rhizobial Nod factor signaling molecules during lateral root base invasion. The chitin-like Nod factor structures are specifically perceived by host-specific receptors. However, the presence of the chitinase-like protein Srchi24 in the proximity of NFs during S. rostrata invasion raised the question whether this protein functions in Nod factor perception or binding during S. rostrata nodulation.

Nodules and lateral roots are both secondary root organs that show common developmental and morphological characteristics, suggesting that nodulation recruited gene functions from the lateral root formation process. The approach to functionally analyze potentially recruited genes was launched in the model system Medicago truncatula. The importance of NAC transcription factors, regulatory SINA proteins, and of small signaling CLE peptides during nodulation is examined in this study and reveals new gene families that presumably evolved to function in nodulation.

This functional study focuses on several aspects of nodulation, Nod factor perception, rhizobial infection, nodule functioning and senescence, which all together increases the molecular knowledge to understand how a nodule can develop and how this is regulated by the plant.
CHAPTER 1

Advances in legume nodulation

Adapted from “Advances in Legume Nodulation”
COVER: Left upper, Medicago truncatula J emalong plant. Right down, Medicago truncatula indeterminate nodule 25 days post inoculation with Sinorhizobium meliloti 1021. The apical white zone of the nodule contains the meristem. Proximal to the meristem the infection zone is formed, in which bacterial uptake takes place, resulting in infected cells where the bacteroids fix nitrogen as observed by the pink color, due to leghemoglobin activity, in the fixation zone.
LEGUME-RHIZOBIUM SYMBIOSIS LEADING TO NITROGEN FIXATION

**Evolution: the origin of legume-Rhizobium interactions**

Root nodule symbiosis (RNS) includes nodulation of legumes and actinorhizal plants; in other words, host species are restricted to the Leguminosae and Fabaceae (for reviews on legume nodulation, see Crespi and Gálvez, 2000; Gage, 2004; Patriarca et al., 2004) and to the actinorhizal plants (eight families of the angiosperms; Benson and Silvester, 1993) and fix N\textsubscript{2} through symbiosis with their microsymbionts, the Gram-negative Rhizobiaceae species and Gram-positive Frankia actinomycetes, respectively, resulting in an additional N\textsubscript{2} source. RNS integrates two distinct programs that can be uncoupled: the bacteria infect the plant tissue to invade the root, and a new organ, a nodule, is initiated, which later becomes occupied by the microsymbiont. The main difference between leguminous and actinorhizal nodules is that the former have a shoot-like anatomy (a peripheral vasculature) with infected cells in the center, developed from a cortical cell-derived primordium, and the latter a root-like anatomy (central vascular bundles), formed from pericycle cell-derived primordia (Gualtieri and Bisseling, 2000). All nodulating plants belong to the Eurosid I clade, including the orders Fabales, Fagales, Cucurbitales and Rosales, with the exception of the symbiosis between Gunnera and cyanobacteria of the genus Nostoc. In this chapter we will focus on the legume-Rhizobium nodulation and its features. Because legume nodulation involves two interlinked and complex processes, namely rhizobial invasion and nodule development, elements have almost certainly been recruited from other symbiotic interactions and plant organ formation, respectively. For instance, the ancient signaling pathway for infection during nodulation is believed to have evolved from the older arbuscular mycorrhization (AM) endosymbiosis. In contrast to nodulation, the majority (80%) of higher land plants form a symbiosis with zygomycete fungi of the order Glomales. This mutualism enables the fungi to grow and complete their life cycle by obtaining carbon from the plant and creates an improved uptake of phosphate from the soil. Appressoria are formed at the root surface, subsequently fungal hyphae penetrate the root to form intracellular arbuscules inside the inner cortical cells, in which uptake of phosphate compounds by the host plant is established (for a recent review, see Harrison, 2005). The comparison and evolution of both endosymbiotic interactions was discussed by Guinel and Geil (2002), who support the model of Schneider et al. (1999) that encompasses a separation of an epidermal and a cortical program of nodulation. Only the epidermal program would have common features with AM and nodulation is assumed to have resulted from two separately evolved programs that over
time came to function together. Whereas a genetic overlap for AM and RNS has been hypothesized, molecular studies resulted in the discovery of several genes that are necessary for root symbiosis with both bacteria and fungi, referred to as the common SYM genes. Mutants in these genes are defective in both symbioses (Nod/Myc). For instance, the NORK gene is part of the common pathway responsible for perception and/or transduction of microbial signals (Kistner and Parniske, 2002; see below) and the ENOD40 and ENOD12 genes are induced by rhizobia as well as fungi (Albrecht et al., 1999). Because comparative studies on both symbiotic interactions can unravel the essential differences and features that lead to one of these biological symbioses, a transcriptomics experiment has recently been performed on AM and nodulated roots, resulting into seven common SYM genes that play a central role in reprogramming of the root for AM and RNS in the model legume Lotus japonicus (Kistner et al., 2005). In conclusion, the overlap of the common genetic pathway of AM and RNS is at the level of intracellular infection.

The other aspect of nodulation, namely initiation of nodule primordia and meristems, has probably also evolved from an existing plant developmental process and has recruited genetic pathways from evolutionarily older organ formations. Considering different plant organ structures, nodules could have originated from, for instance, stem organs, lateral root structures or carbon storage places or sinks. A first indication in support of a lateral root origin is that actinorhizal nodules arise from modified lateral root structures (Newcomb and Wood, 1987). Other indications come from the Medicago truncatula LATD mutant that provides genetic evidence for the evolvement of a nodule meristem from a lateral root meristem (Bright et al., 2005) and from the Rhizobium etli mutant root inducer that initiates root meristems on nodule primordia of Phaseolus vulgaris (common bean) (Ferraioli et al., 2004). Lateral roots and nodules have several features in common: both organs are post-embryonically formed on the root opposite the protoxylem poles, with the development of a meristem and newly initiated vascular tissue. However, the vasculature in a lateral root develops in the center and vascular bundles in legume nodules are formed in a peripheral shoot-like architecture. On the other hand, nodules are carbon sinks where they can fulfill the symbiosis by supplying the rhizobia with sugars; hence, tuber formation programs could contribute to the evolutionary origin of nodule organ formation. Legume-Rhizobium nodulation should be considered as a complex host-specific mutual interaction that results from rhizobial infection via a pathway recruited from mycorrhizal associations and from plant organ formation to host the symbiont, a process recruited from other plant organ initiations.
All together, this life form allows growth and survival of these species in areas where nitrogen supply is limited.

The nodulation process

Rhizobia that grow in the rhizosphere of a host plant attach to the hairs in zone I of the root (i.e. the zone with developing root hairs). The root exudates of legumes contain compounds, such as flavonoids and betaines that are recognized by the bacteria; as a result, the rhizobial NodD protein becomes activated. The NodD proteins regulate the expression of a range of bacterial Nod genes responsible for the synthesis and secretion of the Nod factor (NF) signaling molecules. These NFs comprise four to five β1-4-linked N-acetyl glucosamine residues with a long acyl chain. The NFs from diverse rhizobial species differ in the number of glucosamine residues, the length and saturation of the acyl chain, and the modifications on the chitin-backbone structure, which determine host specificity. Within seconds to minutes upon application of compatible NFs to root hairs, the host plant perception mechanism causes ionic changes in the root hair cells. These changes include a rapid influx of Ca\(^{2+}\), followed by Cl\(^{-}\) and K\(^{+}\) efflux, alkalinization of the cytoplasm, and membrane depolarization. As a consequence, growing root hairs have an increased Ca\(^{2+}\) concentration at the tip and establish a gradient down the root hair. With a lag period of approximately 10 minutes, oscillations in Ca\(^{2+}\) concentration are observed in the cytosolic region associated with the nucleus, a process called Ca\(^{2+}\) spiking (Oldroyd and Downie, 2004). This lag period probably reflects the changes in Ca\(^{2+}\) homeostasis that occurs before spiking, although Ca\(^{2+}\) spiking and Ca\(^{2+}\) flux induction are assumed to be uncoupled. Therefore, both responses might activate different, but possibly overlapping, actions. Following Ca\(^{2+}\) spiking, the root epidermal cells show root hair deformations and branching, which results in newly induced isotropic growth causing the root hair tip to swell and grow in another direction. This modification occurs in the zone of developing root hairs; however, only a few hairs form a tight curl in the presence of NF-producing rhizobia (Geurts et al., 2005; Figure 1A). The bacteria are entrapped in the pocket formed by the curl and locally induced partial cell wall degradation and invagination of the plasma membrane, forming infection threads (ITs) (Geurts et al., 2005; Figure 1B). Rhizobia inside the thread grow and divide, thereby keeping the tubule filled with bacteria. Simultaneously, the first cell divisions are initiated opposite the xylem poles of the root vascular tissue to start the formation of a nodule primordium. During the earliest steps of the M. truncatula-Sinorhizobium melloti symbiosis, microtubular reorganizations are observed in the pericycle and inner cortical cells (Timmers et al., 1999).
Meanwhile, the outer cortical cells prepare for transcellular invasion by rearranging the cytoskeleton and forming cytoplasmic bridges or pre-ITs that will be traversed by the ITs. Finally, inner cortex-derived primordial cells become invaded by ITs (Figure 1C) from which the bacteria are released to differentiate into bacteroids. Prior to the bacterial uptake, infected primordial cells exit the mitotic cycle and go into endoreduplication (Kondorosi and Kondorosi, 2004).

Two types of nodules are distinguished depending on the legume species: indeterminate and determinate. Most legume species, such as M. truncatula, Medicago sativa (alfalfa), Pisum sativum (pea), vetch species and clover species develop indeterminate nodules (Gage, 2004; Figure 2B). The infection zone refers to the zone where bacteria infect the plant cells. Subsequently, nodule zonation is established: a meristematic zone consisting of the middle-cortex-derived primordial cells is installed proximal to the infection zone. The nodule meristem cells have not been traversed by an IT and display the features of meristematic cells, i.e. they are small in size, contain a large nucleus, have a dense cytoplasm, and show active cell division (Timmers et al., 1999). Distal to the infection zone, an interzone and a fixation zone can be distinguished. In the latter, the differentiated plant cells contain the symbiosomes, which are the small factories for nitrogen fixation. At the basal side, a senescence zone arises, where both symbionts degenerate. With the exception of the apical meristem, the other zones are surrounded by lateral nodular tissues, such as the parenchyma, the vascular bundles, and the endodermis. The nodule cortex encloses the lateral tissues and the apical meristem (Vasse et al., 1990). On the other hand, some tropical legumes, such as L. japonicus (Handberg and Stougaard, 1992; Stougaard, 2001), Glycine max (soybean) and P. vulgaris form determinate root nodules (Figure 2A). The nodule primordium originates from the cell cycle reactivation of outer cortical cells that show meristematic activity and become invaded by ITs. Once infected, the primordium becomes
the central tissue of the nodule, which is surrounded by the nodule cortex and nodule parenchyma with peripheral vascular tissue (van de Wiel et al., 1990). After bacterial entrance into the central tissue, cell division stops and the nodule mainly grows because of enlargement of the cells.

In the bacteroids, biological fixation of \( \text{N}_2 \) starts when bacterial nitrogenase enzymes are activated. After the obtained nitrogen compounds are transported to the plant cytosol, nitrate assimilation results in the production of ureids and amino acid components, such as aspartate, glutamate, and amides, essential for further plant growth and survival (Kaiser et al., 1998). Indeterminate nodules of M. truncatula mainly transport amides, such as glutamine and asparagine, to other plant organs (Schoenbeck et al., 2000; Cordoba et al., 2003). Numerous genes involved in ammonium assimilation and asparagine synthesis are induced in L. japonicus (Graham and Vance, 2003), supporting the fact that the amide asparagine is the major transport form of nitrogen exported from the nodules (Vance et al., 1987).

**Alternative infection pathways in water-tolerant legume species**

Legumes have adopted many ‘survival’ methods to colonize extreme environments, such as areas with low nutrients, extremes in water supply and radiation (both light and heat) (Sprent, 2001). For instance, a low nutrient supply is circumvented either by an increase in the root surface area by forming mycorrhizal fungus (ecto- or endo-AM) associations and/or clusters of roots to enhance nutrient uptake in poor soils, or by physiological adaptations, such as nutrient storage in root tuber structures. Another extreme environment is growth in waterlogged soils: the water excess leads to oxygen depletion that retards plant growth, but also inhibits nodulation. As an adaptation to growth in temporarily flooded habitats, some legume species, such as Sesbania rostrata and Discolobium pulchellum, nodulate on the stem when submerged in water (Sprent, 2001). The infection occurs at epidermal cracks on adventitious root primordia present along the stem. The ‘crack entry’ invasion pathway of rhizobia is also observed at lateral root bases (LRB) in a number of tropical legumes, such as S. rostrata and Neptunia plena when roots are grown hydroponically (Goormachtig et al.,
2004b). Under these conditions, accumulating ethylene inhibits zone I root hair formation as well as the root hair curling (RHC) process. Hence, RHC nodulation in zone I, used in well-aerated growing roots, can switch to an alternate ‘crack entry’ nodulation in zone II of the root. During the latter mechanism, bacteria enter the root intercellularly via the formation of infection pockets in the outer cortex of the fissure region. Intercellular ITs grow toward deeper cell layers and progress transcellularly, following a cortical invasion program identical to that described for RHC. The nodulation mechanism of water-tolerant legumes that differs from that of other legumes gives them a great advantage in survival during submergence in nitrogen-limiting soils (Sprent, 2001; Goormachtig et al., 2004a). Another example of an alternative infection is observed in the L. japonicus roothairless 1 mutant (Karas et al., 2005). Here, rhizobial infection embraces crack entry and de novo induction of root hairs from cortical and epidermal cells. Hence, in the absence of root hairs, a secondary track for infection is utilized.

**Nutrition in legumes**

Nitrogen is one of the most important factors that limit plant growth in natural ecosystems and in agricultural systems (in which plants mainly rely on fertilizers for their nitrogen supply). Chemical fertilization often causes environmental problems, such as nitrate loss in the soil and ground water (Lawlor et al., 2001). This problem can be solved by applying nodulated legumes as ‘green manure’ in agriculture and cropping. Biological nitrogen fixation, which reduces N₂ to ammonium, is the most important source of available fixed nitrogen for life on earth. Leguminoseae are the largest family of land plants, and grain legumes provide 33% of the human needs for dietary protein nitrogen. This contribution results not only from their capacity for symbiotic nitrogen fixation (SNF), but also from the production of nutritious protein-rich seeds that make them an essential part of agriculture (Graham and Vance, 2003). SNF involves not only nutrition of the plant by the reduced nitrogen-producing rhizobia, but also insurance of the bacteria of obtaining carbon from the plant. Thus, such mutualism requires exploitation of both plant and bacterial metabolisms. To gather more information on the primary carbon and nitrogen metabolism of plants in general and of the Rhizobium-interacting plants in particular, transcriptomic and metabolomic tools were used by Colebatch et al. (2004) in the model legume L. japonicus. Metabolic pathways, such as glycolysis, CO₂ fixation, amino acid biosynthesis and purine, heme and redox metabolism seemed to be upregulated in nodules compared to those of roots (Colebatch et al., 2004). Genes involved in sugar breakdown were more highly
expressed in nodules than in roots, confirming the observations that sucrose is the primary carbon source for nodule metabolism (Craig et al., 1999). Malate, on the other hand, is probably the direct carbon source of bacteroid metabolism for SNF (Day and Copeland, 1991; Streeter, 1995). Additionally, starch phosphorylases were induced, suggesting a rapid turnover of starch in nodules, and the lack of starch accumulation in functional nodules.

Nitrogenase activity is inhibited by oxygen under aerobic conditions; so, this enzyme needs to be protected from inactivation, which is attained by sequestering the protein in differentiated cells with morphological and biochemical characteristics that limit exposure to oxygen. Such a microaerobic environment is created for the rhizobia in the symbiosomes of the infected cells of nodules. These hypoxic conditions inside the nodule increase the transcription of genes involved in a number of processes, including glycolysis, fermentation, and ethylene biosynthesis (Geigenberger, 2003), in which glycolysis and fermentation prevail as an alternative, albeit less efficient, way to generate adenosyl-tri-phosphate under these conditions.

Additionally, several transporter genes are upregulated in nodules, presumably to assure the high fluxes of sugars into and of amino acids and other nitrogen compounds out of this organ (Rivers et al., 1997; Szczyglowski et al., 1998; Colebatch et al., 2002; Fedorova et al., 2002; Moreau et al., 2002). In addition to the plant, the bacteroids also express a high-affinity inorganic phosphate transporter, essential to SNF (Bardin et al., 1996). By removing this inorganic phosphate from the plant cell, the bacteroids might very probably trigger the synthesis of malate. The hypothesis now states that low cytoplasmic phosphate, together with low free oxygen in nodule cells, may regulate plant glycolysis and CO$_2$ fixation to enhance malate supply for SNF (Colebatch et al., 2004).

**Hormone involvement during nodulation**

Not only the bacterial infection pathway and the nodule type are tightly regulated features of nodulation, but also the position and the number of nodules on the root. All these aspects are principally controlled or strongly influenced by specific phytohormones. Hormonal regulation of the nodulation process is mainly monitored by auxin, cytokinin, and ethylene, but gibberellins and abscisic acid are also involved (Ferguson and Mathesius, 2003).

Both bacterial NFs and flavonoids are known to be inhibitors of auxin transport. After inoculation, a local auxin transport inhibition results in a transient auxin accumulation at the inoculation site, possibly stimulating root cortical cell division and leading to primordium formation (Mathesius et al., 1998). Furthermore, the MtLAX genes, which code for AUX1-like
auxin transporters, are induced during early nodule primordium formation in *M. truncatula*, thereby providing evidence for the need of a certain increase in auxin level at this stage of nodulation, for instance to develop new vascular tissue (de Billy et al., 2001). In addition, the auxin-induced upregulation of the Medsa;CycA2 gene during cell division in nodule formation indicates the need for auxin during meristem establishment (Roudier et al., 2003), while in mature nodules auxin levels decrease. Localization of indole-acetic acid and aldehyde oxidase in the meristem and the infection zone of *M. truncatula* nodules suggests a dual role, in cell division as well as in infection (Fedorova et al., 2005). Whether all the auxin present in the infection zone is synthesized in planta is still unknown; it could also be derived from the prokaryotic symbiont (Lambrecht et al., 2000). Auxin-induced genes are also expressed during the initiation of lateral root development, specifically in the pericycle cells of the root. The formation of both lateral roots and nodules opposite the protoxylem poles seems to be partly a consequence of auxin localization (Mathesius et al., 1998). As a matter of fact, rhizobia have been shown to ‘hijack’ cortical cells at LRBs in zone II of the root to form nodules. This observation suggests that a common response is triggered in cortical cell reactivation for lateral root initiation and nodule formation, whereby rhizobia can infect these ‘activated’ cells when a lateral root field has been created before (Mathesius et al., 2000).

Second, the hormone cytokinin (CK) is known to induce cell division and thereby to initiate meristem formation. Together with auxin, the concentration of this hormone is elevated during the early stages of nodulation and decreases when maturity is reached. Cytokinin responses in root hairs react to rhizobia and CK is present in dividing cells of the primordium (Lohar et al., 2004). CK has a positive effect on the number of nodules in spite of its inhibitory effect on lateral root formation and number. To conclude, auxin and CK that are mainly synthesized in the shoot and root, respectively, are transported throughout the root to create a hormone landscape in space and time leading to a fine-tuned regulation of both organogeneses. In fact, the presence of invading bacteria probably changes local hormone levels that alter the auxin/CK ratio, which is responsible for the induction of cortical cell divisions (Ferguson and Mathesius, 2003).

Also ethylene has been shown to regulate several aspects of nodulation. To start with, it determines the positioning of the nodule with respect to the vascular architecture. The production of a 1-aminocyclopropane-1-carboxylic acid oxidase is induced opposite the phloem poles of the vascular bundle (Heidstra et al., 1997b). The local induction of this enzyme, which is responsible for the last step in the ethylene synthesis, creates a gradient
that inhibits initiation of nodule primordium formation at the phloem poles; hence, nodules are positioned opposite the xylem poles. Also the infection process is inhibited by ethylene at the level of $\text{Ca}^{2+}$ spiking and IT formation (Oldroyd et al., 2001). Hence, the supernodulating sickle mutant presents more infection events due to its insensitivity to ethylene (Penmetsa and Cook, 1997; see section 3.3). In addition, the nodule type is determined by ethylene, as shown for the semi-aquatic tropical legume S. rostrata. In this host, indeterminate nodules are developed in the absence of ethylene (under aerated conditions), whereas determinate nodules are observed in the presence of the hormone (in hydroponic systems) (Fernández-López et al., 1998; Goormachtig et al., 2004b). Finally, ethylene also plays a role during nodule senescence (Sheokand and Brewin, 2003).

Gibberellins (GAs) comprise a large family of over 130 diterpenoid carboxylic acids, some of which with intrinsic growth-promoting activity, but most being precursors or inactivated forms. In the final stage of GA biosynthesis, these forms are converted to bioactive GAs by GA20-oxidase (GA20ox) (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). Until recently, the role of GAs during nodulation was only speculative, based on the observations in L. japonicus that nodules contain higher levels of GAs than roots and that by applying exogenous GA node-like structures could be induced, probably by stimulating cell division and cell elongation (Ferguson and Mathesius, 2003). A role for GAs during the nodulation process has only been studied in S. rostrata (Lievens et al., 2005) and in P. sativum mutants (Ferguson et al., 2005; see below). In S. rostrata, GAs are involved in intercellular invasion at the LRB. A gene coding for an active GA20ox, SrGA20ox, is transiently upregulated in an NF-dependent manner, downstream from the NF signaling. SrGA20ox transcript localization is observed in cells surrounding infection pockets and young parts of the ITs. Thus, GAs may be produced during intercellular invasion and could be essential for infection pocket and IT formation. LRB nodulation under hydroponic conditions results in determinate nodule formation because of the ethylene accumulation, which also promotes LRB, but inhibits RHC nodulation (Goormachtig et al., 2004a). GAs may act in the same manner to influence infection and invasion because GAs inhibit infection of rhizobia via RHC but promote LRB intercellular invasion under water-logged conditions (Lievens et al., 2005). A second expression pattern for SrGA20ox was detected in the pre-infection zone of the central tissue, more specifically in differentiating cells derived from the nodule meristem (Lievens et al., 2005). This pattern resembles the expression of GA20ox detected at the periphery of the meristem during shoot apical meristem (SAM) and leaf development, which is possibly involved in cellular differentiation (Vogler and Kuhlemeier, 2003).
Cell cycle activation and organ patterning during nodulation

Several physiological and molecular signals are connected to determine the position at which the cell cycle is re-activated and whether a meristem could be established. These signaling mechanisms involve the synthesis, transport, and degradation of hormones, resulting in a spatial and temporal hormonal landscape that acts together or is coupled with the activation of transcription factors and other regulatory proteins. The overall process of signals and cellular changes leading to the formation of a particular organ at a certain position in the plant is called ‘organ patterning’. Aspects determining the nodule ‘patterning program’ and the differentiation of the primordial cells into ‘nodule-specific’ tissues are described here.

Simultaneously with the infection of root hairs by rhizobia, the root cortical cells (and pericycle cells) opposite the protoxylem poles of the vascular tissue are re-activated for cell division and re-enter the cell cycle to proceed to the G1-S transition state. In the outer cortical cells, pre-infection threads are formed to allow the ITs to pass through and end up in the inner cortex-derived nodule primordium. During indeterminate nodulation, meristematic cells arise proximal to the infected cells that activate several cell cycle genes, such as histones and cyclins, e.g. Medsa;CycA2 (Roudier et al., 2003) and Sesro;CycB1;1 (Goormachtig et al., 1997). Determination of cell fate includes the expression of other genes, such as regulatory transcription factors, and the KNOX homeodomain protein. In general, the function of KNOX proteins during normal plant development has been tightly correlated with regulation of polar auxin transport (Tsiantis et al., 1999), which regulates patterning of organ development, and elevated CK levels (Frugis et al., 1999). Recently, research on the KNOX protein of Arabidopsis thaliana revealed that this transcription factor activates CK synthesis, which is necessary for cell division in the SAM (Jasinski et al., 2005), in contrast to inhibition of the GA synthesis by repression of GA20ox expression. Differentiating cells undergo enlargement, which could be regulated by GAs. Thus, appearance of GA20ox may be associated to differentiation, such as the formation of leaf primordia (Barley and Waites, 2002) or nodule primordia. Indications for the latter directly rise from the isolation of a KNOX homolog in M. truncatula (Koltai et al., 2001), which possibly inhibits GA production to keep a meristem identity in the indeterminate nodules of M. truncatula.

Further, the expression of ENOD40 is induced by an increased CK level and is essential for cortical cell division (ccd) and nodule primordium formation. In conclusion, the model for SAM development involves KNOX activity to initiate meristematic tissue through the production of CKs, but also inhibition of GA20ox, and of GA synthesis as well.
On the other hand, the infected cells of the nodule are known to go into endoreduplication, i.e. division arrests. The genome of the bacteroid-containing cells becomes duplicated and these polyploid nodule cells increase in cell size. The CCS52 protein, which is a substrate-specific activator of the anaphase-promoting complex ubiquitin ligase, is produced in these cells and regulates the initiation of endoreduplication (Vinardell et al., 2003; Kondorosi and Kondorosi, 2004).

Finally, active auxin transport concerns the expression of PIN-FORMED auxin efflux (Friml et al., 2003) and AUX1-like LAX auxin import carriers (Schnabel and Frugoli, 2004) during nodule development. As a result, the spatial and temporal landscape of auxin concentrations at the nodule initiation probably determines the patterning of nodule sites, likewise as in other higher plants (Friml, 2003; Reinhardt et al., 2003).

**Genetic approaches to discover the key nodulation functions**

To unravel the biological and molecular aspects leading to symbiotic nitrogen fixation, the biotechnological research field continues to develop sophisticated techniques, thereby creating the opportunity to resolve the key regulatory mechanisms in a relatively fast way. In the following parts, we will give a detailed overview on the biotechnological techniques utilized in legume species and the future potential to exploit this research onto non-legumes and agriculturally important crops.

**GENOME-WIDE APPROACHES AND BIOTECHNOLOGY IN MODEL SYSTEMS**

**Phylogeny**

Legumes or Fabaceae are among the best studied plant families in the world. For several reasons, this interest is logical: they include 650 genera and 18,000 species, forming the third largest family of higher plants; they have symbiotic relationships with rhizobia that produce high quantities of biological nitrogen, turning them into a major source of organic fertilizer; they symbiotically interact with fungi; they maintain high protein production in their seeds (20-40%), which turns them into the main source of vegetable protein in human diets and livestock feed; and they produce beneficial secondary compounds (Young et al., 2003). Phylogenetical classification divides legumes into three subfamilies, Mimosoideae, Caesalpinoideae and Papilionoideae (Figure 3). The latter, which involves most cultivated legumes, consists of four clades: the genistoid clade (including Lupinus), the aeschynomene/dalbergioid clade (the peanut), the clade that comprises two subclades of
a common ancestor, the Hologalegina: the Loteae (L. japonicus) and the ‘temperate’ or ‘galegoid’ legumes (e.g. Melilotus, Trifolium, Medicago, Pisum, Vicia, Cicer and Lens) and finally, the phaseoloid clade consisting of the ‘tropical’ or ‘phaseoloid’ legumes (e.g. Phaseolus, Vigna, Glycine, and Cajanus) (Gepts et al., 2005). However, Mimosoideae, with Acacia and Albizia members and Caesalpinoideae, with Poinciana (flame tree) and Tamarindus (source of tamarind fruit) members, are also important to people and forest vegetation (Young et al., 2003). Comparative analysis of the legume genomes reveals that synteny is high among closely related species, but declines with increasing phylogenetic distance and is significantly more convoluted between the galegoid and phaseoloid clades (Choi et al., 2004).

Model legumes
Agricultural crop legumes, such as soybean, bean, pea and lentil, are relatively poor model systems for genetic and genomic research. The difficulties to study these legumes are their large and/or duplicated genomes, their recalcitrance for transformation, their long generation time and/or their large seedlings that prevent high-density culture (Udvardi et al., 2005). As a result, M. truncatula (Barker et al., 1990; Cook, 1999) and L. japonicus (Handberg and Stougaard, 1992) have been internationally adopted as model legume systems to study the molecular basis with the ultimate aim to transfer knowledge to and to improve agronomic traits of grain legumes, such as seed quality and pest resistance. M.
truncatula and L. japonicus have been chosen as the models for indeterminate and determinate nodule formation, respectively (Cook, 1999), because of their small diploid genome (470-550 Mbp) (Barker et al., 1990; Ito et al., 2000), their self-fertility, short seed-to-seed regeneration time, and their transformability (Trieu and Harrison, 1996; Trinh et al., 1998; Aoki et al., 2002).

M. truncatula Gaertn. or barrel medic originates from the Mediterranean area and is used as a feed stock in permanent pastures of the subtropical areas of Australia and the USA. A number of natural populations that exhibit a high level of polymorphism have been collected (Prosperi et al., 2001). M. truncatula is the most appropriate model for legume crops, such as pea, faba bean (Vicia faba), chickpea (Cicer arietinum), clovers (Trifolium sp.), and lucerne (M. sativa), all members of the Galegoid phylogenetic group (Choi et al., 2004).

The Japanese name of L. japonicus is “Miyakogusa”, meaning “capital weed”, possibly because the weed was common in Kyoto or the bright and showy color of its flowers reminded of the luxury of the capital city. Plants collected on a riverbank in Gifu were launched as a valuable tool for legume research. The research activity of L. japonicus in Japan is mostly carried out by a nonprofit organization, the Miyakogusa Consortium, which keeps track of linkage maps, expression arrays and transformation techniques. The L. japonicus Seed Center was set up at the National Agricultural Research Center for the Hokkaido Region and recombinant inbred lines are distributed (Kawaguchi et al., 2001).

**Comparative genome and sequence analysis**

The big genome sizes of some legumes result mainly from genome duplications and abundant retro-elements, associated with extensive C methylation. In G. max, for instance, a high level of microsynteny between several bacterial artificial chromosome (BAC) contigs suggests a recent large-scale duplication event (Yan et al., 2003). High levels of microsynteny have been found between both model legume species and agricultural legumes, including M. sativa (Endre et al., 2002), P. sativum (Gualtieri et al., 2002) and G. max (Yan et al., 2003; Young et al., 2003). Also, M. truncatula and A. thaliana genomes share an eroded network of microsynteny, but no macrosyntenic relationships are apparent between the two models (Zhu et al., 2003; Yan et al., 2004). This divergence in genome nature reflects the incapability of A. thaliana to accomplish SNF interactions. In conclusion, legumes or Fabaceae are now assumed to be a coherent taxonomic group with substantial genome conservation, which makes it not only possible to extend legume genomic resources
Chapter 1

across taxonomic boundaries, but also to reduce the genome fraction that needs to be sequenced to discover most genes. The Legume Information System is a comparative legume resource that integrates genetic and molecular data from multiple legume species, developed by the National Center for Genome Resources (http://www.comparative-legumes.org; Table 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Genotype size (Mbp)</th>
<th>N</th>
<th>Tribe</th>
<th>Clade</th>
<th>SL</th>
<th>PL</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. truncatula</td>
<td>Barrel medic</td>
<td>500</td>
<td>8</td>
<td>Trifoleae</td>
<td>Galegoid</td>
<td>183</td>
<td>130</td>
<td>A17, A20, DZA</td>
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<tr>
<td>M. sativa</td>
<td>Alfalfa</td>
<td>1600</td>
<td>16</td>
<td>Trifoleae</td>
<td>Galegoid</td>
<td>70</td>
<td>68</td>
<td>Mscw2, Mscq93</td>
</tr>
<tr>
<td>P. sativum</td>
<td>Pea</td>
<td>5000</td>
<td>7</td>
<td>Viceae</td>
<td>Galegoid</td>
<td>101</td>
<td>68</td>
<td>J115, J1281, J1339, J1194</td>
</tr>
<tr>
<td>G. max</td>
<td>Soybean</td>
<td>1100</td>
<td>20</td>
<td>Phaseoleae</td>
<td>Phaseoloid</td>
<td>56</td>
<td>15</td>
<td>PI209322, Evans</td>
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<tr>
<td>V. radiata</td>
<td>Mung bean</td>
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<td>11</td>
<td>Phaseoleae</td>
<td>Phaseoloid</td>
<td>62</td>
<td>31</td>
<td>TC1966, VC3890</td>
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<tr>
<td>P. vulgaris</td>
<td>Common bean</td>
<td>620</td>
<td>11</td>
<td>Phaseoleae</td>
<td>Phaseoloid</td>
<td>37</td>
<td>22</td>
<td>BAT93, Jalo</td>
</tr>
<tr>
<td>L. japonicus</td>
<td>Bird's foot trefoil</td>
<td>500</td>
<td>6</td>
<td>Loteae</td>
<td></td>
<td>67</td>
<td>44</td>
<td>L. filicaulis, L. japonicus Gifu</td>
</tr>
</tbody>
</table>

**TABLE 1:** Attributes of species used for synteny analysis.
N, gametic chromosome number; SL, sequenced loci; PL, polymorphic loci. Reprinted from Choi et al. Proc Natl Acad Sci USA 101, 15289-15294; ©2004, with permission.

M. truncatula has a diploid genome (2x 8 chromosomes) with a small size (500-550 Mb). Nearly 50% of the DNA is highly condensed (heterochromatic) and localized around the centromeres, whereas the remaining DNA is present in the chromosome arms where it is less condensed (euchromatic); these euchromatic arms contain the majority of the genes. Thus, these gene-rich regions gained first priority in the genome sequencing project. The construction of a physical map of the M. truncatula genome was initiated by D. Cook (University of California, Davis), and will be useful for positional cloning, quantitative trait locus analysis, genome sequencing and comparison to other legume genomes. The M. truncatula genome is sequenced by several participants: the University of Oklahoma, the Samuel Roberts Noble Foundation, the US National Science Foundation (www.genome.ou.edu/medicago.html), the European Union 6th Framework “Grain Legume Integrated Project” Program, for chromosomes 3 and 5 (http://medicago.toulouse.inra.fr/EU/ChrV/), and other laboratories. For a full description of the M. truncatula sequencing project, see www.medicago.org/genome. L. japonicus Gifu and
Miyakojima genome sizes were determined to be 494 and 512 Mb, respectively and contain twice six chromosomes, which are sequenced in the Kazusa DNA Research Institute (http://www.kazusa.or.jp/eng/index.html).

Most genes are present in the euchromatic region of the genome; thus, the construction of genome libraries of M. truncatula and L. japonicus, as BACs that are rich in genes would be expected to cover most genes. These large insert clones could be anchored to extensive map resources that are available in M. truncatula (mtgenome.ucdavis.edu) and L. japonicus to create a contiguous assembly of genome sequences that comprise almost all of the genes, the so-called ‘gene space’. For M. truncatula and L. japonicus the gene space is 270 Mb and 230 Mbp with a gene density of 1 gene/6.7 kb and 1 gene/6.3 kb, respectively. Assuming that there are 35,000 to 40,000 genes overall, sequencing of 230 to 270 Mb will reveal essentially all genes (Young et al., 2005). The BAC clones are ready for direct sequencing and for mutant analysis. The latter includes mutant complementation by subcloning of the BAC, which contains the target gene, into a binary vector and transformation of the mutant. For L. japonicus Gifu and M. truncatula a library of 30,720 BAC clones, with an average insert size of 140 kb and of 102 kb (Nam et al., 1999) is constructed, respectively. Together with the correlating amplified fragment length polymorphism bands on the BAC clones, a full genome-assorted contig of the model legumes is being constructed that will fulfill the genome sequencing needs. The contig map of M. truncatula covers approximately 480 Mb or 95% of the genome. Mapped clones are sequenced at the Oklahoma University (B. Roe). A genome database for M. truncatula is created as “URMELDB” (M. Spannagl, www.urmeldb.net). To provide the community access to M. truncatula genome annotation, more information is provided on a series of web pages via http://www.tigr.org/tdb/e2k1/mta1/ (IMGAG Annotation, GbrowseBACView, ContigPipeline, GeneOntology, MedicagoGeneIndex, and Sequence Search). On the other hand, M. truncatula expressed-sequence-tag (EST) sequencing is daily updated on The Institute for Genome Research database web site (http://www.tigr.org/tdb/tgi/mtgi), which groups ESTs into contigs to produce tentative consensus sequences (TCs). The European EST databases are accessible at http://medicago.toulouse.inra.fr/Mt/EST (Journet et al., 2002). Furthermore, the MtDB groups all public M. truncatula ESTs into approximately 17,000 contigs plus a similar number of singletons (Lamblin et al., 2003); http://www.medicago.org/MtDB). L. japonicus gene space sequencing is covered for 90% today and captures 90% of the ESTs in the database. This advance facilitates fast gene
discovery as closely linked molecular markers are quickly located onto sequenced BAC clones, which allow the identification of candidate genes (S. Sato, pers. comm.).

G. max is one of the major and globally important legume crops, but because of its large polyploidy and complex genome (1,115 Mb), it is not favored as a model. Nevertheless, extensive mutant analysis, physical mapping, comparative and functional genomics approaches are being performed on soybean, because a deeper understanding of its genome organization and evolution is essential for successful breeding and continued genetic and biological research (Stacey et al., 2004). Sequencing of EST libraries was initiated in 1998 by The Public Soybean EST Project and microarray and serial analysis of gene expression as well as BAC library and genome sequencing is going on. The Soybean Genetics Executive Committee creates, organizes and evaluates all data, an effort that should lead to a complete and full analysis of the soybean genome (the SoyBase Web, http://129.186.26.94/).

**Tools: bioinformatics, transcriptomics, proteomics, and metabolomics**

The release of the complete A. thaliana, rice and poplar genome sequences resulted in a major breakthrough in bioinformatical analyses and in silico predictions to resolve various biological questions. Although the genome sequencing of M. truncatula, L. japonicus and G. max is not yet finalized, very interesting and indicative research has been performed with the aim at broadening our insight into ‘special’ properties of legumes, consequently offering great opportunities to improve legume crops (Stacey and VandenBosch, 2005). A few gene families specific and unique to legumes have already been identified: 2,525 legume-specific EST contigs have been obtained by computational analysis of legume (M. truncatula, L. japonicus and G. max) in comparison to non-legume (Arabidopsis, rice, tomato, etc.) unigene sets (Graham et al., 2004). Less than 3% of these legume-specific ESTs showed clear homology to previously characterized legume genes. With the aim to predict gene functions, single-linkage clustering and motif analysis of related sequences identified gene families with conserved motifs, such as defensin-like cysteine cluster proteins, and proline-rich and F-box-related proteins. The identified motifs are represented across diverse taxa, indicating that these are fast-evolving genes. This finding confirms the hypothesis that a fraction of the legume-specific genes had non-legume origins, but have diverged so much they now appear unique to legumes. On the contrary, the true novel sequences in legumes may concern families too small to analyze their motifs (Graham et al., 2004). One example of legume-specific proteins includes a family of small cysteine-rich peptides found through in
silico transcriptome analysis on cDNA libraries of M. truncatula nodules. This peptide family, the NCR peptides, is specific for the Galegoid species (Mergaert et al., 2003). Furthermore, to unravel gene functions that had specifically been acquired by legumes to act during nodulation, in silico analysis was performed on M. truncatula EST nodule libraries. This study revealed the existence of 340 genes that are solely expressed in nodules (including a fraction of 2.6% of the total annotated TCs (Fedorova et al., 2002). Of the nodule-specific genes, 31% show strong homology to sequences from non-legume species, supporting the hypothesis that genes were recruited from pathways common to plants. In addition, half of these 340 TCs that appear to be legume specific encode most of the nodulin and leghemoglobin proteins as well as cysteine cluster proteins and calmodulin-like proteins (Fedorova et al., 2002). In conclusion, an in-depth bioinformatical study would supply a lot of useful information for further research, although the predictions should be treated with care.

An excellent example is the previously characterized legume-specific proteins, the legume (leg)hemoglobins (Lbs), with nodule-specific function that do not appear to be unique for legumes. During legume nodulation, symbiotic Lb proteins are produced (providing the nodules a blood-red color), and are proposed to function as oxygen transporters, with an extremely fast O₂ association and a relatively low dissociation rate (Appleby, 1984). This feature ensures an adequate supply of oxygen for bacterial respiration at low concentration of free oxygen that prevails in infected cells, avoiding damage to the oxygen-sensitive rhizobial nitrogenase enzyme within the nodule tissue. Recently, symbiotic Lb RNA interference (RNAi) experiments performed in L. japonicus have proven the essential role of Lbs in SNF (Ott et al., 2005).

However, a common evolutionary origin has been suggested among the animal hemoglobin and leghemoglobin proteins because of their structural similarities (Bogusz et al., 1988), implying that hemoglobin has been inherited from a common ancestor and, thus, might be present in all plants, and not only in nodulating plants, such as the legumes and the actinorhizal plants. Experimental evidence came from the identification of hemoglobin genes in both the nitrogen-fixing non-legume Parasponia andersonii and in the related non-nitrogen fixing non-legume Trema tomentosa (Bogusz et al., 1988; Bogusz et al., 1990). A root-specific promoter activity was observed in transgenic tobacco (Nicotiana tabacum), suggesting a role in the respiratory metabolism of root cells. Transgenic Lotus corniculatus had a nodule-specific promoter activity, with induction of this non-legume hemoglobin in the central bacteroid-containing portion of the nodule, indicating that the
regulatory signals necessary for nodule-specific expression of hemoglobins are conserved between different legume species (Marcker et al., 1984; Stougaard et al., 1987). In addition, also non-symbiotic hemoglobins have been identified in both legumes and non-legumes that are structurally similar to human hemoglobins, neuroglobin, and histoglobin (Kundu et al., 2003) and are possibly implicated in a plant function, such as detoxification via the binding to NO, hereby influencing defense responses (Downie, 2005). In conclusion, the question has risen whether an adaptation of the hemoglobins was required during nodule evolution, making Lbs legume-specific gene products (Bogusz et al., 1990).

The first step to validate in silico data includes transcript profiling. The existing tools cover a range of different reliability, sensitivity, time/labor-consuming and function-indicative factors. Examples of a number of techniques applied in legume biotechnological research, in order of sensitivity, are macro- and microarrays, cDNA-AFLP, and the extremely sensitive Affymetrix chip hybridization. Also for M. truncatula, this ‘transcript-covering’ chip is available and consists of 549 phase-3 BACs (12,685 sequences), chloroplast phase-2 and phase-3 BACs (8,876 sequences), The Institute for Genomic Research MtGl 8.0 (TCs/singletons) (33,550 sequences), 6,500 M. sativa ESTs (1,895 sequences) and S. meliloti 1021 genome (8,405 sequences) (G. May, pers. comm.).

Finally, we have to mention that the Noble Foundation (Ardmore, Oklahoma) and the European FP6 framework are generating important programs of transcriptomics, proteomics, and metabolite profiling to facilitate the use of all kinds of recently created tools in the legume genome world (www.noble.org/medicago/index.htm; http://medicago.toulouse.inra.fr/Mt/EST). The M. truncatula site for collected data of plant cell culture, microarray, SAGE, proteomics and metabolomics is http://medicago.vbi.vt.edu/. To conclude, all major links to any legume database available at this moment can be found at http://www.comparative-legumes.org/lisg/lis_links.

**Mutagenesis of model legume plants**

Although sequence information and gene expression analyses enable us to speculate about gene function, the definitive allocation of function requires the introduction of genetic mutations. Forward genetics is basically used to identify mutants that are defective in a specific process, which is followed by map-based or transcript-based cloning of the mutant gene. Finally, complementation with the wild-type gene proves functionality. On the other hand, reverse genetics may reveal the role of a specific gene of interest. In the end, the
detailed analysis of mutant collections will allow a broader but more fine-tuned insight into the different steps resulting in nitrogen fixation.

Many different approaches have been developed to generate mutant populations of M. truncatula (Tadege et al., 2005) and L. japonicus (Udvardi et al., 2005). For instance, chemical (ethyl methanesulfonate (EMS)) mutagenesis causes a high density of point mutations in the genome and provides allelic series for functional analysis. These mutants are used for forward genetic screening, followed by classical map-based cloning. A recently developed reverse genetics strategy designated targeting-induced local lesions in genomes (TILLING; Colbert et al., 2001) allows the identification of specific gene mutations in an EMS-mutagenized population by the application of PCR (Perry et al., 2003; www.lotusjaponicus.org/finder.htm; EUFP6 Grain Legume Integrated Project Tilling Initiative). Another technique to generate mutagenized populations uses ionizing radiation (fast neutrons and X-rays) to produce deletions, ranging from a few base pairs up to more than 30 kb, and chromosomal rearrangements that result in knockout mutants. Both forward and reverse genetic screens are performed to analyze the mutant population. Furthermore, transfer-DNA (T-DNA) tagging and (retro)transposon insertion disrupt genes by insertional mutagenesis. Cloning of the tagged gene is relatively easy in these mutants because flanking sequence databases are available. However, obtaining genome-wide coverage of insertion mutants is less straightforward, because this strategy relies on the size of the gene and the genome. Last, RNAi or post-transcriptional gene silencing is applied for transcript knockdown in reverse genetics, but requires transformation and tissue culture. Different collections of mutants in M. truncatula, L. japonicus and G. max allow screening of different kinds of nodule phenotypes. Examples of classes of gene function being analyzed are very early NF responses (perception and signal transduction mutants), infection/invasion features, meristem determination/maintenance, hormone perception, autoregulation of nodulation (AON) and nodule pathways for functional symbiosis. In the following sections, the major mutants unraveling these functions will be discussed.

**DISCOVERY OF THE KEY GENES**

**Perception of nodulation signals**
Legumes perceive the rhizobial NF signaling molecules at very low concentrations (up to $10^{12}$ M) and the establishment of a symbiotic interaction relies on precise NF structural requirements. However, the question how this symbiosis is exactly accomplished and by
which mechanism(s) remains unsolved. In the ’90s, the first candidates included different classes of proteins capable of binding NFs. Two NF-binding site proteins, NFBS1 and NFBS2, were isolated from root extracts of M. truncatula and cell suspension cultures of M. varia, respectively (Bono et al., 1995; Niebel et al., 1997). NFBS1 has a low binding affinity, lacks NF structure specificity, and is produced in the presence of combined nitrogen, suggesting that this protein does not uniquely function as a symbiosis mediator, whereas NFBS2 has high binding affinity and NF structure specificity, making it a more likely symbiosis-specific protein (Gressent et al., 1999). Recently, a high-affinity NFBS3 protein has been identified in M. truncatula roots and, in contrast to NFBS1 and NFBS2, depends on the common symbiotic genes DMI1 and DMI2 (Hogg et al., 2006). Second, a lectin nucleotide phosphohydrolase was isolated from Dolichus biflorus roots (Etzler et al., 1999). Finally, several chitinase-like enzymes were identified and have been proposed to be involved in NF perception (Goormachtig et al., 2001; van der Holst et al., 2001). Chitinases degrade chitin molecules and, because NFs have a chitin-backbone structure, these proteins may probably bind to NFs and function as receptors or adaptors to concentrate the signal molecules around the plant receptors. Finally, different genes encoding potential NF-binding proteins whose expression is upregulated upon rhizobial inoculation have been found in legume species, but until today, no symbiotic role during the NF perception by the host plant could be shown (Gressent et al., 2002). In parallel, an approach was launched to discover the key genes for perception of NFs, including the mapping of mutants without any normal nodulation responses. Several of these mutants led to the identification of the ‘real’ receptors, with the potential to bind NFs or to form a complex with adaptor proteins to sense the nodulation signals.

In the legumes M. truncatula, M. sativa, L. japonicus and P. sativum, different receptor-like kinases (RLKs) have been identified by mutant analysis. These RLKs include members of two classes: with leucine-rich-repeats (LRR) or with LysM-containing extracellular domains (Figure 4). By the study of bacterial nodulation mutants, the ‘entry/signaling’ receptor model has been proposed (Ardourel et al., 1994; Figure 5) that together with the recent findings straightened out the NF perception pathway. The model states that two kinds of receptors are involved in the establishment of a symbiotic recognition pathway. On the one hand, mapping of mutants completely lacking NF-induced responses led to the discovery of genes (encoding receptors) required to set the nodulation process in motion, the so-called NF ‘signaling receptors’; on the other hand, receptors with a
higher stringency for the specific NF structure and later action in the interaction, the so-called NF ‘entry receptors’ assume the strict host specificity of the interaction.

**FIGURE 4:** Overview of receptor kinases involved in nodule initiation via root hair curl invasion along with the processes they might control. LysM-type receptor kinases might bind the nodulation factor, could work as (hetero)dimers and might interact with leucine-rich-repeat (LRR) receptors, whose ligands are unknown. PsSYM2, MtLYK3 and MtLYK4; LjNFR5, MNFP and PsSYM10; MsNORK, MIDM12, LjSYMRK and PsSYM19 are orthologs. Reprinted from Goormachtig S, Capoen W, Holsters MG (2004) Trends in Plant Science 9, 518-522. ©2004, with permission from Elsevier.

Signaling receptor candidates are LjNFR5 (Madsen et al., 2003), MtNFP (Amor et al., 2003) and PsSYM10 that encode LysM receptor kinases and are orthologous to each other. Transcripts were found in uninoculated roots and the mutant plants lacked any NF response upon inoculation. In contrast, mycorrhizal symbiosis is normal, indicating that the receptor acts independently of the pathway shared for fungal endosymbiotic systems. The LysM receptor kinases contain three LysM domains (domains previously found to bind peptidoglycans and chitin; (Bateman and Bycroft, 2000) that possibly have NF binding capacity, and a serine/threonine kinase domain, without auto-activation loop, which is atypical for serine/threonine kinases. Another RLK, LjNFR1, with two LysM domains and a serine/threonine kinase domain including the activation loop makes it a potential activator of NFR5 by phosphorylation (Radutoiu et al., 2003). Probably, one of the receptor-like kinase LYK genes in M. truncatula provides the ortholog of NFR1, but a loss-of-function mutant,
such as nfr1 in L. japonicus, is not isolated yet, which can be the consequence of a genetic redundancy.

The stringent demand of the entry receptor candidates for a specific NF structure has first been observed in the PsSYM2 gene (Geurts et al., 1997). Synteny-based cloning in M. truncatula resulted in the identification of two LysM domains that contain receptor-like kinases, LYK3 and LYK4, which are involved in IT formation (Limpens et al., 2003). By RNAi knockdown analysis, root hair curling and microcolony formation of rhizobia were found to still occur, but without formation of ITs. Furthermore, strong alleles of LYK3 had multiple root hair outgrowths and swelling, but no curling. The same phenotype has previously been described for the EMS mutant hcl that has turned out to have a mutation in the LYK3 gene (Catoira et al., 2001, T. Bisseling, pers. comm.). The microtubular cytoskeleton network is probably reorganized as a result of re-initiation of root hair growth with deficient ability to establish curling. In these mutants, expression of ENOD11 (an early nodulin gene involved in (pre-)infection; (Journet et al., 2001)) and NIN (an early nodulin required for IT and nodule primordium formation; Schauer et al., 1999; see below) is normal, favoring the model that LYK3 is an entry receptor and not a signaling receptor. Moreover, the epidermal NF responses, which are still induced in the hcl mutant, could be uncoupled from infection and ccd; these two responses are not induced in this mutant. LYK3 and LYK4 are highly homologous to the LjNFR1 gene, but loss-of-function phenotypes are different.

Other components, ‘the Doesn’t Make Infections1’ (DMI1), DMI2 and DMI3 in M. truncatula act at or upstream of Ca\textsuperscript{2+} spiking and are required for the symbiotic interaction with AM fungi as well as for nodulation. DMI mutants apparently perceive NFs by the same mechanism that leads to root hair branching as does the wild type, but the DMI1 and DMI2 mutants respond by root hair swelling without production of branches and are defective in Ca\textsuperscript{2+} spiking (Catoira et al., 2000; Wais et al., 2000). The identification of these three genes means that the common pathway for mycorrhizal and rhizobial infection, triggered by a ‘fungal factor’ and the ‘Nod factor’, respectively, involves a specific Ca\textsuperscript{2+}-dependent intracellular secondary signaling prior to microorganism infection of the plant. DMI1 is located on chromosome 2 and encodes a 883 amino acid protein that contains four transmembrane domains (two leucine-Zipper domains and a proline-rich domain, suggesting protein-protein interactions) and a ligand-gated cation channel domain. Together with DMI2, DMI1 is required for NF-induced Ca\textsuperscript{2+} spiking. The gene is constitutively expressed in roots and lower transcript levels were detected in pods, flowers, leaves, and stems. Inoculation with rhizobia did not affect the transcript level. DNA gel blotting revealed the presence of
orthologs in lots of other leguminous species, such as *L. japonicus* (Pollux), *P. sativum* (Sym8), and *S. rostrata*. Candidate proteins to interact include LYK3 and LYK4, NFR1 and NFR5, NFP, and DMI2 and DMI3. Because of a high degree of conservation for the orthologs among angiosperms and because only a single gene with high similarity is present in rice and *A. thaliana*, the molecular and biological function of the DMI1 protein seem very conserved and nodulation apparently arose from an ancient, conserved pathway for mycorrhizal associations (Ané et al., 2004).

Dudley and Long (1989) described the non-nodulating alfalfa mutant MN-1008, which was defective in both RHC and in ccd, supporting the idea that these distinct cellular processes are triggered by integrated mechanisms. The gene coding for a nodulation receptor kinase NORK in *M. sativa* (corresponding to DMI2 in *M. truncatula*), was mapped (Endre et al., 2002) and was revealed to be an LRR-RLK. Later on, the NORK ortholog in *L. japonicus* (SYMRK) was cloned (Stracke et al., 2002). The proteins possess an amino-terminal signal peptide, an extracellular domain containing three LRR motifs, a transmembrane segment, and an intracellular serine/threonine kinase domain. The presence of approximately 50 different genomic NORK extracellular sequence-like domains in the *A. thaliana* genome indicates a large family that contains this motif; however, these NORK extracellular sequence-like domains could not be identified in animals, lower eukaryotes, or prokaryotes. The only NF response observed in the NORK mutants was root hair deformation. Ca$^{2+}$ spiking, which induces further responses, such as ccd and gene expression, was absent. The combined absence of Ca$^{2+}$ spiking and of NF-dependent induction of ENOD11 suggested an early split in the NF-induced signaling cascade upstream of DMI2/NORK. Later on, Limpens et al. (2005) observed that a weak allele of DMI2 allows rhizobial infection, but bulbous ITs and almost no bacterial uptake results in inefficient invasion. Hence, NF signaling - via DMI2 - is necessary for maintenance of infection and uptake of bacteria in plant cells.

Additionally, the NORK mutants of the legume species *M. truncatula* (DMI2), *M. sativa* (NORK) and *L. japonicus* (SYMRK) were found to be responsible for an enhanced touch response in the root hairs (Esseling et al., 2004). Root hairs of DMI2 mutants respond with curling to NF application, but the curling stops when the root hair touches its own shank and entrapment of the bacteria is blocked. Finally, the study on SrSymRK in *S. rostrata* showed that when the epidermal responses and touch responses were circumvented by the crack entry invasion under hydroponic conditions, RNAi of SrSymRK results in a phenotype similar to the weak allele of the DMI2 mutant, including a defective uptake of rhizobia (Capoen et
DMI2 seems to play a role during biological events that all include a touch response mechanism and Ca\(^{2+}\) spiking. Because DMI2 is required at the heart of endosymbiosis in leguminous plants to release bacteria into nodule cells during a process that might testify of common origins between bacterial and fungal endophytic lifestyles, DMI2 functions probably in a complex for symbiosis-induced secondary signaling.

The third gene, the *M. truncatula* DMI3 gene, acts downstream of Ca\(^{2+}\) spiking, because nodulation responses are blocked after Ca\(^{2+}\) spiking in the mutant lines. DMI3 was mapped on chromosome 8 and was identified as a calcium-calmodulin-dependent protein kinase through transcript-based cloning (Lévy et al., 2004; Mitra et al., 2004). The full-length open reading frame encodes a 523 amino acid protein that contains an N-terminal kinase domain, common with other plant calcium-dependent protein kinases, and a C-terminal calcium-binding regulatory domain similar to the mammalian visinin-like domain (with three calcium-binding EF hands). In between both domains a calmodulin-binding domain is located that overlaps an autoregulatory domain, allowing kinase activity to be regulated by both calcium and calmodulin. DMI3 is only expressed in root tissues and a green fluorescent protein (GFP) fusion localizes in the nucleus of epidermal root cells (Kaló et al., 2005). The identification of DMI3 as a putative calcium-sensitive effector protein thus confirms the role of calcium as an integral part of both the NF signal transduction pathway as well as the mycorrhizal signaling cascade. Because DMI2 and DMI1 mutants are still able to generate a calcium influx response, but not a further downstream signaling, Ca\(^{2+}\) spiking, and not the calcium influx, might be the signature recognized by DMI3. Further downstream activation of the pathway is assumed by the phosphorylation activity of the activated calcium-calmodulin-dependent protein kinase.

The first committed steps for nodule development downstream of the common pathway with mycorrhization are represented by the primary transcription factors NSP1 and NSP2 (Catoira et al., 2000; Oldroyd and Long, 2003). Mutant plants have a Nod\(^{-}/\)Myc\(^{+}\) phenotype, lacking NF-dependent responses, such as rhizobial infection, ccd, and gene expression, whereas reduced root hair deformations are still observed and NF-induced Ca\(^{2+}\) spiking is retained. NSP1 and NSP2 encode GRAS-like (Scarecrow-like gene; Pysh et al., 1999) transcription factors, which are in general involved in plant developmental processes and are found throughout the plant kingdom (33 members in *A. thaliana*). The NSP proteins contain a variable N-terminal region and a GRAS domain that consists of two leucine-rich regions that might indicate protein-protein interactions. The N-terminal domain contains homopolymeric stretches of amino acids also found in the activation domain of other
transcription factors. NSP1, which is mapped on chromosome 8, has only 17% identity and 32% similarity with NSP2, suggesting that they fulfill similar, but non-redundant, functions. The PsSym7 mutant has a similar phenotype, thus is a potential ortholog of NSP2. NSP1 probably evolved from a family of transcription factors with a non-symbiotic function, whereas highly homologous sequences to NSP1 are found in non-legumes, such as poplar (two putative proteins), A. thaliana (Scarecrow-like 29) and rice (putative protein OsHNO). Additionally, NSP1 is conserved and could have obtained an additional function in NF signaling during the evolution of legume species. The nsp1-2 allele that lacks the C-terminal SAW motif causes a strong nodulation phenotype, indicating that this motif is essential for its functioning. NSP1 is constitutively expressed in roots and a GFP-fusion driven by the NSP1 promoter region reveals nuclear localization in root epidermal and cortical cells. Because DMI3 colocalizes with NSP1 in the nucleus of uninoculated plants and genetic analysis shows that DMI3 acts directly upstream of NSP1, NSP1 is proposed to be a target of DMI3 (Smit et al., 2005). Also NSP2 maps on the M. truncatula genome, namely on chromosome 3. In addition to induction upon inoculation of the roots (4 dpi, 1.6-fold; 7dpi, 3.2-fold), NSP2 is also expressed in shoots and leaves, although no phenotype was obtained in the mutant. A C-terminal GFP-fusion driven by a 35S promoter localizes to the nuclear envelope and (more weakly) to the endoplasmic reticulum. However, the GFP signal shifts to the nucleus upon inoculation, whereas for NSP1 a constant nuclear localization was observed. Additionally, a Cameleon transgenic line, which is a stable transgenic line of M. truncatula expressing a calcium reporter construct based on fluorescence resonance energy transfer interaction and fluorescence fluctuations upon different Ca$^{2+}$ concentrations in the cell, detects Ca$^{2+}$ spiking in the cytosol and in the nucleus, indicating that Ca$^{2+}$ spiking acts in NF signal transduction in the nucleus. So, DMI3 probably activates NSP2 in the nucleus, indirectly or directly via phosphorylation (Kaló et al., 2005).

More downstream from these primary transcription factors, the nodule inception (nin) mutant was the first mutant characterized in L. japonicus. Cloning of this Ac-transposon-tagged mutant locus resulted in the identification of a potential transcription factor (Schauser et al., 1999). Upon inoculation with Mesorhizobium loti, the mutated plants show ‘shepherd’s crook’ structures accompanied by excessive root hair deformations and curling of individual root hairs. The infection zone of the root is extended, but no IT formation or primordium initiation occurs. The phenotype indicates that Nin is involved in the initiation of root nodules, and that the gene acts before IT initiation and cortical cell division. Expression is upregulated upon inoculation, primarily in the primordial cells of a
developing nodule, although transcripts were also observed in mature nodules, namely in the parenchyma, vasculature, and central tissue of young pink nodules (20 dpi), suggesting an additional function later in nodulation. The protein contains 878 amino acids and consists of several domains identified previously in transcriptional regulators, so NIN is a potential transcription factor for nodulation acting downstream of the early signal exchange between the symbionts.

In summary, the earliest molecular signaling components and mechanisms during nodulation are not totally clear yet, although the last few years there has been an enormous evolution in the discovery of essential key regulators of these very early steps. The study on two model legume species has advantages as well as disadvantages to unravel the pathway. The complexity of the LysM receptor family in different species might already be an explanation on the variability of NF perception. For instance, the question whether LYK3 or HCL is the M. truncatula ortholog of LjNFR1 is still open. A probable cause of differences is the redundancy level of genes of these model systems, which also develop a different type of nodules.
Invasion of rhizobia

Besides the very early genes necessary to trigger the nodulation program in legumes, other genes are involved in sustained efficient rhizobial infection, in maintenance of the NF responses, or in bacterial uptake and nodule differentiation. The mutants described below are characterized by aberrant or defective infection, invasion or uptake of rhizobia and provide more information on the molecular requirements and biological functioning during nodulation.

First, the L. japonicus Snf mutants (including four loci of two types) nodulate in the absence of rhizobia, resulting in pseudonodes. Snf gene expression is not suppressed upon inoculation of the roots, but is inhibited by nitrate. Grafting experiments with wild-type and mutant shoots and roots vice versa reveal a root-based mechanism. Snf1 acts independently from NFR1, so Snf1 could be part of the NF signaling pathway by acting between the receptor complex NFR5/NFR1 and the NIN function. Other Snf genes could suppress the ccd (J. Stougaard, unpublished results).

Second, nine mutants have been isolated in L. japonicus that are all defective in root hair development and have a concomitant low nodulation phenotype (Wopereis et al., 2000). These mutants were classified in four categories: root hairless (Rhl), short root hair (Srh), petite root hair (Prh), and variable root hair (Vrh). Upon inoculation, the Rhl1 mutant does not show ITs or ccd, although the other three mutants still form ITs (K. Szczyglowski, pers. comm.). Inoculation of the double mutant Rhl1/HAR1 results in more nodule primordia on the roots, whereas the single Rhl1 mutant plants develop fewer nodule primordia compared to HAR1, a mutant defective in AON; thus, Rhl1 does not interfere with the AON pathway. The absence of root hairs obliges the Rhl mutant to use two pathways that differ from the default RHC infection, namely ‘crack-entry’ and cortical cell-derived root hair infection. As a result, the Rhl1 mutant is delayed in nodulation and a potential function of RHL1 concerns the de novo induction of root hairs from epidermal and cortical cells that is partially taken over by NFs in the mutant (Karas et al., 2005). These results strengthen the hypothesis that root hairs by themselves are not required for the cortical program during nodulation.

Another mutant in M. truncatula, designated LATD, shows defects in lateral root and nodule development (Bright et al., 2005). The root phenotype is described as the formation of a primary root that gradually ceases to grow and that develops abnormal tips. Similarly, the initiation of lateral roots looks normal, but these structures also remain short with abnormal tips. Upon inoculation with S. meliloti, infection occurs and nodule primordium formation is initiated, but infection is rarely completed and as a result, immature non-fixing
nodule organs are formed. In addition, the rhizobia that invaded the nodule lack the expression of bacterial genes necessary for differentiation and nitrogen fixation. So, apparently plant and bacterial development are blocked in LATD mutants, but plants are Myc⁺, indicating that this gene is not required for symbiosis per se, but functions in root, lateral root and nodule meristem maintenance that is essential for nodule development (Bright et al., 2005).

The numerous infections with polyphenolics (nip) and sluggish infection (sli) mutants were described by (Veereshlingam et al., 2004): both show infection nodulation phenotypes. The SLI mutant does not only have sluggish infections, but also the shoot looks somewhat stunted. Both genes are believed to be involved in suppression of defense responses elicited during rhizobial infection (R. Dickstein, pers. comm.). During invasion of NIP1 mutant nodule primordia, the rhizobia seem to be confined to ITs, which is reminiscent of the DMI2 weak allele phenotype, except for the autofluorescence, indicating accumulation of polyphenolic compounds in the nodule. Furthermore, electron microscopy shows the lack of bacterial release, and no expression of ENOD8 (a symbiosome marker) was seen. Other evidence for abnormal gene expression was given by induction of a phenylalanine lyase defense gene and the absence of late nodulin transcripts. The nodulation phenotype together with the fact that abnormal lateral roots are developed in this mutant is remarkably similar to that of the LATD mutant, although NIP1 could not complement the latter mutant (R. Dickstein, pers. comm.).

How important are the persistence of infections and nodule differentiation leading to nitrogen-fixing nodule organs is illustrated in the M. truncatula EMS-induced LIN mutant (Kuppusamy et al., 2004). Plants mutated in this gene have fewer infections upon inoculation with rhizobia because of a bacterial arrest in the epidermis, resulting in nodule primordium, but not in mature nodule formation. Several markers for nodule differentiation, such as CSS52a, N6, ENOD2, and ENOD8 were not expressed. This finding supports the hypothesis that IT persistence and invasion are necessary for differentiation and meristem maintenance.

Many more mutants are currently being characterized in the model legumes that will finally result in the identification of several marker genes for the different stages during nodulation. Not only plant gene expression, but also rhizobial gene mutations associated to nodulation have the capacity to induce defects during all phases of the symbiotic interaction (Mitra et al., 2004). An example of a bacterial mutant that induces meristem identity defects of the legume nodule has been described (Ferraioli et al., 2004). Determinate nodules of P.
vulgaris develop upon inoculation with Rhizobium vulgaris, but when the bacterial Tn5 mutant, called root inducer (RIND), was used for inoculation, all early events during the infection process were normal; at later stages, roots originated from the nodule primordia and additional ectopic roots developed. The latter were closely spaced and were agravitropic, showing anastomosis. Apparently, because of the mutation in the rhizobia, the ITs aborted early inside the root hair without induction of late nodulin genes as a consequence, resulting firstly in the absence of a differentiated central tissue of the globose nodules and secondly in increase of root meristems and formation of aberrant roots from the nodule primordium. These observations indicate a potential root-specific pro-meristematic cell identity for the nodule primordium in P. vulgaris.

**Hormone regulation during nodulation**

Because most plant hormones act at different levels during plant growth and development, the study of their effects is very complex as well as the understanding of hormone perception and regulation during nodule formation. Some knowledge has been obtained by analysis of expression patterns of hormone-responsive promoter-GUS lines and pharmacological experiments on legume roots. In addition, the application of mutants defective in hormone regulation or perception could also unravel hormone involvement in nodulation. Unfortunately, only a few mutants that affect nodulation due to hormone defects have been found yet.

The most important and best studied is an ethylene-insensitive EMS mutant in M. truncatula named sickle, for its unusual sickle-shaped zone of nodulation (Penmetsa and Cook, 1997). Recently, map-based cloning showed that the gene mutated in sickle codes for the EIN2 ethylene receptor gene of M. truncatula (P. Gresshoff, unpublished results). This mutant displays a supernodulating phenotype whereas inhibition of rhizobial infection in the susceptible root zone is lost. The infection efficiency of wild-type M. truncatula is low, with 3% to 8% of the infections persisting to colonize a nodule organ. On the contrary, the sickle mutation affects the number of persistent infections without altering the transient root susceptibility, resulting in an increase in infection number relative to that of wild type (Penmetsa and Cook, 1997). Ethylene has been implicated as the secondary signal in the inhibition of nodulation by both nitrate and light (Ligero et al., 1991); thus, ethylene might be an endogenous signal for regulation of rhizobial infection, because of its influence on the number of infectable root hair cells. In addition to the nodulation phenotype, a root
phenotype has been observed that clearly proves that shifts in hormone balances influence a wide range of plant processes.

Recently, Ooki et al. (2005) have characterized the L. japonicus LOT1 mutant with ethylene defects for nodulation responses. The mutant has a reduced nodule number, up to 20% of that of the wild type, caused by a repression of IT formation, albeit normal looking root hairs. Once nodule primordia were formed, nodules were always functional. A possible explanation for this nodule phenotype could be related either to the higher sensitivity to exogenous nitrate or to the occurrence of a nodulation-specific ethylene-dependent pathway that is constantly activated in this mutant. In addition, distorted trichomes and dwarfism were also observed. This pleiotropic phenotype indicates that LOT1 functions in local regulation of nodule control as well as in trichome formation and growth.

Reduction of the level of GAs and brassinosteroids (BRs) causes shoot dwarfism, and both hormones influence nodulation in a positive manner. In GA-deficient mutants of P. sativum nodule organogenesis is reduced. Grafts of mutant and wild-type shoots or roots again restored nodule numbers, as well as application of exogenous GA (up to $10^{-6}$ M) could bring the phenotype of the mutant back to wild type. However, adding a high concentration ($10^{-3}$ M) of exogenous GA becomes inhibitory (Lievens et al., 2004; Ferguson et al., 2005). Previously, BRs were not considered to affect nodulation, but BR-deficient mutants of P. sativum were shown to influence the nodule number as well. For GAs, the level of hormones in the root is crucial, but for BRs the shoot level of hormones seemingly controls nodule numbers in an indirect shoot-mechanism-dependent manner. Other observations in these GA and BR mutants are related to lateral root numbers, which are strongly correlated with the nodule number. These findings not only suggest an overlap in the hormone-dependent early developmental pathways of both organs, but fits with the fact that phytohormones have a broad range of effects on plant organogenesis (Ferguson et al., 2005).

**Autoregulation of nodulation**

Because nitrogen fixation and photosynthesis are both energy-demanding processes, the number of nodules is controlled and optimized by the plant. A tight control occurs when fixed nitrogen is easily available in the soil, i.e. nitrate inhibition of nodulation, but also in the absence of nitrate, when the AON mechanism is at work. Initiated nodule primordia send a signal to the leaf, which in turn produces an inhibitory feedback signal to the root that blocks further nodule formation on young root tissue (Pierce and Bauer, 1983; Kosselak and Bohlool, 1984). In a split-root system, which comprises the separation of the roots of an
intact plant in two compartments, rhizobial inoculation of one half of the root system partially blocks subsequent nodulation of the other half. This suppression did not result from a decreased number of infections, but rather from an increase in the number of aborted infections (Caetano-Anollés and Gresshoff, 1991). In fact, the autoregulatory effect is triggered by a pre-nodulation event, because nodulation has already been suppressed when one side of a split-root system is pretreated with NFs (van Brussel et al., 2002). The model proposes that autoregulation consists of a root-derived signal generated at the nodule initiation sites and a shoot-derived autoregulatory signal produced in shoots, mainly in the leaflets (Gresshoff, 2003). Legume species have possibly recruited genes that control the stem cell proliferation in the SAM to extend this regulatory mechanism to the root for maintenance of homeostasis during nodulation.

AON involves RLKs that are found in large plant families, of which some appear to be involved in peptide signaling (Boller, 2005). Known genes responsible for AON include HAR1 in L. japonicus (Nishimura et al., 2002), NTS1 (GmNARK) in G. max (Searle et al., 2003) and SUNN in M. truncatula (Penmetsa et al., 2003). These genes encode LRR-RLKs and are expressed in shoots and in nodulated roots indicating long-distance functioning because of the perception of signals traveling between the shoot and the root. These genes are homologs of the A. thaliana CLAVATA1 (CLV1) gene, which forms a receptor-complex with CLV2 (both LRR-RLKs) that is thought to interact with the CLV3 peptide (Matsubayashi, 2003). Hence, the question arises whether a CLV3-like interactor is the signal peptide shuffling between the root and the shoot, although CLV1 has not been implicated in long-distance signaling. However, evidence for CLV3/ESR-like (CLE) peptides functioning as morphogens in A. thaliana supports a potential role for members of the CLE family or other small peptides during AON, in which formation of the nodule organ or primordium is prevented (Boller, 2005).

The mRNA level of GmNARK was 14-fold higher in leaves than in the SAMs and also HAR1 transcripts were not detected in shoot apices, together supporting the idea of nodulation control via the leaf. The hypernodulating mutants have an increased number of nodules, not restricted to zone I that is caused by the loss of the long-distance signaling from roots via leaves (Wopereis et al., 2000; Searle et al., 2003). Grafting experiments provided evidence for the responsibility of the shoot genotype in the negative regulation of the root nodulation (Nishimura et al., 2002). Also, an altered root phenotype (more lateral roots and a shorter root) was observed, especially in the absence of rhizobia, hereby
indicating the link to a similar autoregulation mechanism in lateral root development (Caetano-Anollés and Gresshoff, 1991).

In M. truncatula, the SUNN mutant also displays the hypernodulating phenotype and, again, grafting proved the shoot-derived regulation of nodule organogenesis. Although the short root is not accompanied by an increased number of lateral roots, in contrast to the observations in HAR1 and NTS1 mutants (Penmetsa et al., 2003), this variability in root phenotype may reflect differences in species context. The study on the potentially interacting proteins of SUNN in M. truncatula resulted in several candidate genes, named RLP1, LSS, RDN, and RAE (J. Frugoli, unpublished results). RLP1 is mainly expressed in leaves and encodes a receptor-like protein without kinase domain. The gene has 95% nucleotide identity to SUNN in the extracellular part (Schnabel et al., 2005). A second candidate gene is LSS that cannot complement the Sunn phenotype, but the gene is tightly linked to SUNN, indicating it is a potential interactor. Thirdly, the RDN mutant phenotype suggests that the RDN gene could encode a root-determinate factor for AON. Finally, the RAE mutant is a suppressor of supernodulation, restoring AON but not the root phenotype.

Another aspect to consider regards the involvement of shoot-derived auxin in AON signaling. Auxin transport has been thought to be slower in the autoregulation-deficient SUNN mutant than in wild-type M. truncatula. However, this impression was a consequence of the shorter cortical cells of the SUNN roots in comparison to the length of wild type root cortical cell (Van Noorden et al., 2006). The relative speed is equal to the speed of the shoot-root auxin transport of the wild type. Furthermore, at 24 h post inoculation, the time point for the onset of AON, the nodule numbers and the long-distance auxin transport from the shoot decreased in wild-type but not in SUNN mutant plants. SUNN mutant plants fail to reduce the auxin amount loaded from the shoot, leading to supernodulation. This is in contrast with the ‘auxin burst control of nodulation’ hypothesis, which can still be correct in determinate nodulation. This hypothesis states that, subsequent to the initial induction or ‘burst’ of nodule primordia, shoot-derived auxin export into the root is stimulated, resulting in elevated auxin levels that inhibit further nodule primordium initiation and, as such, control nodule numbers (Gresshoff, 1993). Finally, a local auxin transport inhibition induced by the rhizobia is needed to initiate nodule formation and also occurs in SUNN mutants, whereas at the onset of AON the long-distance auxin-signaling reduces the auxin flow to the root in wild type, but does not change in SUNN mutants (Van Noorden et al., 2006).

AON signaling in determinate and indeterminate nodulation seems to differ as well. The arrest of nodule formation in M. truncatula and P. sativum (indeterminate) occurs...
before nodule primordium formation, while G. max and L. japonicus (determinate), in which aborted primordia are found, only arrest further nodulation after nodule primordium formation (P. Gresshoff, pers. comm.). Additionally, recent grafting experiments indicated that in indeterminate nodule-forming legumes, such as M. truncatula, an additional shoot-derived factor may be required for symbiotic progression compared to determinate nodulation (Lohar and VandenBosch, 2005).

CONCLUSIONS AND APPLICATIONS

Because nodulated legumes are not limited by nitrogen and because they are capable of accumulating remarkable levels of proteins, legume crops are of enormous agricultural significance. Grain legumes can contain up to threefold more proteins than cereals. Nearly 33% of all human nutritional requirements for nitrogen come from legumes and in many developing countries, legumes serve as the single most important source of proteins. A major advantage of the application of nitrogen-fixing legumes to produce plant proteins is a substantial decrease in the consumption of fossil fuels (necessary for nitrate synthesis), thereby lowering the agricultural contribution to global warming. Moreover, growing legumes in agricultural rotations promotes diversity and leads to a decrease in plant pathogen populations, resulting in a reduced need for pesticides. Besides their advantages as grain legumes, legumes act as green manure for the subsequent crop on the field, thanks to the capacity of biological nitrogen fixation. Thus, legumes are extremely important in the development of sustainable agricultural systems (Drinkwater et al., 1998).

Therefore, the interest of the scientific community to the model system M. truncatula is also a matter of advances in agriculture, besides the fundamental biological interest in legume nodulation processes. The European FP5 and FP6 framework projects (in collaboration with the USA) specifically apply the knowledge of model legume systems to improve grain legume agriculture.

Because a high level of synteny has been detected by comparative genomics between the model systems, M. truncatula and L. japonicus, and other legume crops, such as soybean, and because nodulation is a principal feature in the nitrogen balance for an ecologically sustainable world, research on the nitrogen fixation properties of these biological model systems will be of huge relevance in the future.
So, the study of the molecular pathways that specifically results in the nodulation capacity of legumes, such as the perception, invasion and fixation mechanisms, in parallel with the hormonal landscapes and the plant control mechanisms, not only fulfills the fundamental knowledge on nodulation signaling pathways, but also creates the feasibility of future extensions to non-legume crops. For instance, rice comprises several interesting properties to study the potential of non-legume crop nodulation (Dey et al., 2004; Giraud and Fleischman, 2004).

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CHAPTER 2

Signaling and gene expression for water-tolerant legume nodulation

Adapted from “Signaling and Gene Expression for Water Tolerant Legume Nodulation”
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COVER: upper left image, Sesbania rostrata nodulated stem. Middle lower image, close-up of S.rostrata plants in the field.
AQUATIC LEGUMES

Biological reduction of molecular dinitrogen to ammonium provides most of the natural nitrogen input for life on earth. Plants of the legume family (Leguminoseae) acquire such fixed nitrogen through a symbiotic interaction with Gram-negative soil bacteria, called "rhizobia", of which most belong to the Rhizobiaceae. Leguminoseae is the largest family of land plants, and grain legumes provide 33% of the human needs for dietary proteins (Graham and Vance, 2003). As a consequence of this symbiosis, new root organs are formed, the nodules, in which bacteria reside under appropriate conditions to fix nitrogen. In most legumes, including the model species Medicago truncatula L. Gaertn. and Lotus japonicus R. Larsen, nodules develop in the region just above the root tip where root hairs grow (Figure 1). Rhizobia attach to the growing root hairs and induce swelling, deformation, and curling of the hairs. In the curl, a microcolony is entrapped and local cell wall hydrolysis and ingestion of the cellular membrane results in infection threads (ITs) that contain a matrix in which the bacteria reside and penetrate the cortex transcellularly via cytoplasmic bridges, also designated preinfection threads. ITs are targeted toward the cells of the nodule primordium (NP) that develops in the cortex. Ultimately, the bacteria are released in some cells of the incipient nodule and differentiate into bacteroids, the factories for nitrogen fixation (reviewed by Crespi and Gálvez, 2000; Patriarca et al., 2004; Figure 1).

![Figure 1: Synthetic scheme of the root hair curling (RHC) (left) and lateral root base (LRB) (right) invasion pathways on S. rostrata roots. Stereomicroscopic images of nodules formed through RHC invasion (left) and LRB invasion of A. caulinosans (expressing a β-glucuronidase marker) on S. rostrata roots. During RHC invasion, the bacteria are entrapped within the curl and induce the formation of an intracellular infection thread (it) within the root hair. The infection proceeds intracellularly towards the cells of the nodule primordium (np) where the plant cells take up the bacteria. During LRB invasion, the bacteria (orange) colonize the intercellular spaces between the cortical cells and induce the formation of infection pockets (ip), from which intercellular and intracellular infection threads (it) guide the bacteria to the target cells for nitrogen fixation within the nodule primordium (np). Important traits of both invasion pathways that differ are indicated by purple text (modified from Goormacheling et al., 2004a).](image-url)
In waterlogged soils, root hair curl (RHC) invasion is arrested because accumulating ethylene inhibits both root hair formation and the RHC process (Sprent, 2001; Goormachtig et al., 2004a). However, in flooded ecosystems, nitrogen fixation may provide an additional advantage because leaching, increasing denitrification, and the low nitrogen input via mineralization of organic matter render the soils extremely poor in nitrogen accessible for the vegetation (James et al., 2001; Koponen et al., 2003). Aquatic legume species have evolved variations of the "classical" nodule formation pathway to allow nodulation in flooded habitats.

*Sesbania rostrata* Brem (higher Papilionoideae) is the model plant to study aquatic nodulation. It is an annual (semi-)tropical legume, indigenous to the Sahel region of West-Africa where it occupies temporarily flooded areas. The plant establishes an efficient symbiosis with *Azorhizobium caulinodans* ORS571 (Dreyfus et al., 1988) and is used as green manure for rice cultivation. Otherwise, the legume has no nutritional value for people or cattle and no breeding has been performed. *Sesbania rostrata* has a diploid genome with $2n = 12$ chromosomes, corresponding to a DNA content of 1187 Mbp (Le Coq et al., 1985).

As an adaptive trait to life in a desert with occasional periods of submergence, *S. rostrata* has along its stem several rows of adventitious root primordia with an apical meristem and a stem-connected vascular bundle (Figure 2A). Upon submergence, the meristem is reactivated and gives rise to an adventitious root (Figure 2B) (Duhoux and Dreyfus, 1982; Caturla et al., 2002). After inoculation with *A. caulinodans*, the adventitious root primordia turn into nodules (Figure 2C). The bacteria enter directly into the root cortex via a fissure that is formed because of the protrusion of the adventitious roots. This invasion mode, designated 'crack entry' or lateral root base (LRB) invasion, is a typical trait of aquatic nodule formation (Goormachtig et al., 2004a). Thus, the *S. rostrata* "stem" nodules are actually adventitious root nodules, because the vascular system of the nodule is connected to the vascular tissue of the adventitious rootlet, and not directly to that of the stem (Duhoux, 1984; Goormachtig et al., 1997, 1998b). Adventitious roots can still develop upon submergence of mature stem nodules because the apical root meristem of the dormant adventitious root is preserved and probably arrested in early S phase (Duhoux and Dreyfus, 1982; Goormachtig et al., 1997, 1998b). Another unique feature of stem nodules on *S. rostrata* and some *Aeschynomene* sp. that differentiates them from root-borne nodules is the presence of chloroplasts in the nodule parenchyma and cortex, allowing photosynthesis. Moreover, this photosynthetic activity might contribute to the very high nitrogen fixation rates reported in these plants (Evans et al., 1990; Hungria et al., 1992; James et al., 1998). Other so-called stem nodule-developing legumes include other *Sesbania* sp., *Aeschynomene* sp., *Neptunia* sp., *Discolobium pulchellum* Benth.,
Lotus uliginosus Schkuhr., and Vigna lasiocarpa (Mart. ex Benth.) Verdc. (Alazard and Duhoux, 1990; Loureiro et al., 1994, 1995; Subba-Rao et al., 1995; Boivin et al., 1997; James and Sprent, 1999; James et al., 2001). The stem nodulation feature has also been described in some actinorhizal plants (Prin et al., 1992).

Sesbania rostrata also forms nodules on its underground root system. In aerated soils, RHC invasion takes place in the region of developing root hairs (Goormachtig et al., 2004b), while under submergence, the nodules appear at the LRBs (Figure 1). The bacteria enter the cortex via cracks caused by the lateral root protrusion, similarly to what happens on the stem. This alternative invasion pathway, which involves infection pocket (IP) formation, is also observed in Neptunia plena (L.) Benth. (Goormachtig et al., 2004b), Stylonsanthes sp. (Chandler et al., 1982), Aeschynomene sp. (Alazard and Duhoux, 1990; Loureiro et al., 1995), Neptunia natans (L.) Druce (Subba-Rao et al., 1995), Arachis sp. (Boogerd and van Rossum 1997), and Chamaecytisus proliferus (L.) Link (Vega-Hernández et al., 2001).

Stem nodule development is a good system to hunt for early nodulin genes because of its synchrony (Goormachtig et al., 1998b; Lievens et al., 2001). On the other hand, various pharmacological and physiological studies have provided an insight into LRB nodulation. Here, we give an overview of aquatic nodulation.

**LATERAL ROOT BASE INVASION**

Upon sensing the exuded S. rostrata flavonoid liquiritigenin (Goethals et al., 1990; Messens et al., 1991), a signal exchange and recognition between the two symbiotic partners initiates LRB invasion: nodulation (nod) genes of A. caulinodans are induced to synthetize the Nod factors (NFs), decorated lipochitooligosaccharides, which are secreted and perceived by the plant to induce nodulation (D'Haeze and Holsters, 2002). To colonize the outer cortex, the bacteria
enter through cracks in the epidermis (Figure 3A) and induce a local plant cell death via NF signaling. Some plant cells in the invasion region have disrupted cell walls, lose their plasma membrane integrity, and are characterized by vacuole fragmentation and electron-dense precipitates in the cytoplasm (Ndoye et al., 1994; Goormachtig et al., 1997; D’Haeze et al., 2003). The collapse of a few cells creates intercellular spaces for the formation of IPs that are occupied by bacteria (D’Haeze et al., 1998, 2003). ITs initiated from IPs lead the bacteria toward the NP (Figure 3B). Cortical cells that flank IPs and intercellular ITs show signs of cell wall fortification and accumulation of phenolic compounds, features reminiscent of pathogen infection (Parniske, 2000). The cortical invasion proceeds further as described for the RHC infection (Figure 1). NF-triggered cell cycle reactivation of the root inner cortical cells triggers the formation of an NP (Goormachtig et al., 1998b). When the ITs reach the NP, the bacteria are released into the plant cells and a fixation zone is created consisting of infected cells interspersed by some uninfected cells. Distal from the infection zone, a meristem is established that delivers new cells to the developing nodule. At this stage, the NP has a 360° open basket-shaped structure that continues to grow because of the distally located peripheral meristem (Figure 3C). Clearly, the S. rostrata nodules are of the indeterminate type. While infection progresses and the nodules grow, the open-basket architecture creates the impression that the developing nodules "engulf" the bacteria with an infection zone moving more distally, following the meristem. Finally, the meristem activity ceases and the nodule size increases by enlargement of central tissue cells (Figure 3D) (Ndoye et al., 1994; Goormachtig et al., 1998b). The developing nodules have an indeterminate character, although an early arrest in the nodule meristem results in round-shaped determinated mature nodules on hydroponic roots.

As mentioned above, adventitious root nodules on the stem and LRB nodules follow the same developmental track, but the former have a periderm that derives from a concentric secondary meristem at the outside of the developing nodule (Goormachtig et al., 1998b). The periderm might function as a protective barrier against oxygen but might also hinder photosynthetically generated O₂ from escaping, the reason for increased nitrogen fixation in stem nodules upon exposure to high irradiances (James et al., 1998). Additionally, an enhanced presence of glycoproteins in the cell wall and intercellular spaces of parenchyma and cortical cells that are more densely packed than in root nodules would restrict oxygen diffusion, possibly by cross-linking through reactive oxygen species (ROS) activity, generated within nearby chloroplasts (James et al., 1996a). Transcripts of the S. rostrata early nodulin 2 gene (SrEnod2) are localized in the nodule parenchyma and in nodule tissue surrounding the
connecting vascular tissue. The parenchyma-specific expression of the SrEnod2 nodule is not determined by its promoter or 5’ untranslated region, but through the cis elements located in the 3’ untranslated region (Chen et al., 1998). SrEnod2, which is a hydroxyproline-rich cell wall protein, has been proposed to contribute to the formation of the oxygen barrier (van de Wiel et al., 1990).

During adventitious root nodulation, several primordia are often initiated per rootlet, resulting either in multilobed nodules, in which the lobes are separated by parenchyma or in a fused ring of central tissue around the central vascular bundle of the original root primordium (Goormachtig et al., 1998b).

FIGURE 3: Schematic representation of the adventitious root nodule development of Sesbania rostrata (A, B, C, and D) and in situ localization studies with 35S-labeled antisense RNA probes for some genes expressed upon inoculation with Azorhizobium caulinodans (E to L). Nodulation stages are: 1 to 3 days post inoculation (dpi) (A), 3 to 4 dpi (B), 4 to 6 dpi (C), and 6 to 8 dpi (D). Infections pockets are in gray; periderm in red; nodule parenchyma with vascular bundle in green; central tissue infected by A. caulinodans in dark blue; dots, uninfected cells; I, meristem zone; II, invasion zone; III, fixation zone. The in situ localizations are presented as dark-field pictures (signals seen as white spots). In situ localization of SrIb6 (E) and SrSAMS (I) on longitudinal sections (10 µm) through developing adventitious root nodules at 2 dpi, of SrENOD40 (F) and SrPme1 (J) at 3 dpi, of Srchi24 (G) and SrSAMS (K) at 4 dpi, and of SrENOD2 (H) and SrIb3 (L) at 6 dpi; rp, root primordium; ic, infection center; ip, infection pocket; iz, infection zone; npa, nodule parenchyma; np, nodule primordium; nv, nodule vascular bundle; star, distal meristem cells; 1, 2, and 3, different concentric zones containing various quantities of SrENOD40 transcripts. Bar= 0.1 mm.
NOD FACTOR PERCEPTION DURING LRB INVASION

Nod factors are indispensable for both RHC and LRB nodulation, but the structural requirements are more stringent for RHC invasion (Goormachtig et al., 2004b). NFs of A. caulinoedans ORS571 mainly consist of chitopentasaccharides, carrying a palmitic (C16:0), vaccenic (C18:1), or stearic (C18:0) N-acyl chain, with an N-methyl and a 6-O-carbamoyl group at the non-reducing end (Mergaert et al., 1993, 1997a, 1997b). The reducing end remains unsubstituted or bears a D-arabinosyl or an L-fucosyl substitution on C3 and C6, respectively (Mergaert et al., 1993, 1997b, 1997c; D’Haeze and Holsters 2002). Mutant bacteria producing aberrant NFs that lack decorations can still nodulate S. rostrata via LRB invasion, although nodulation is delayed and inefficient. This effect becomes progressively worse with the synergistic absence of NF substituents. Under conditions of RHC infection, these mutant bacteria induce root hair deformation and cortical cell division, but not infection and functional nodulation (D’Haeze et al., 2000; Goormachtig et al., 2004b).

NFs act at concentrations as low as $10^{-9}$ to $10^{-12}$ M, are perceived through specific receptors (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003), and are mainly located in the cell walls of root hair and other epidermal cells and, at high concentrations, also in the plasma membrane (Philip-Hollingsworth et al., 1997; Goedhart et al., 2000). Candidate receptors capable of binding NFs and of downstream signaling are plasma membrane proteins found in the root epidermis and synthesized during nodule development. (Oldroyd and Downie, 2004; Geurts et al., 2005).

In the NF "entry/signaling" receptor model proposed by Ardourel et al. (1994), two kinds of receptors are required to establish a symbiotic recognition pathway to set the nodulation process in motion, the NF "signaling" and "entry" receptors. Several classes of (putative) NF receptor or binding proteins have been identified in different legume species (Oldroyd and Downie, 2004; Geurts et al., 2005).

Mapping of mutants that completely lack NF-induced responses in the legumes L. japonicus and Pismum sativum (L.) cv. Sparkle, led to the discovery of orthologous receptor-like kinases (RLKs), LjNFR5 and PsSYM10, respectively (Madsen et al., 2003), whereas MtNFP (Amor et al., 2003) is suspected to be the ortholog in M. truncatula (Table 1). These genes encode LysM receptor kinases that contain three LysM domains (previously found to bind peptidoglycans and chitin; Bateman and Bycroft, 2000) with a possible NF-binding capacity and a serine/threonine kinase domain without typical auto-activation loop. Mycorrhizal symbiosis is normal, indicating that the receptor acts independently of pathways shared with...
fungal endosymbiotic systems (Amore et al., 2003; Madsen et al., 2003). LjNFR1 codes for another RLK with two LysM domains and a serine/threonine kinase domain, including the activation loop that potentially activates NFR5 by phosphorylation (Radutoiu et al., 2003; Table 1).

The stringent demand of the entry receptor candidates for a specific NF structure has first been observed in the PsSYM2 gene (Geurts et al., 1997). Synteny-based cloning in M. truncatula identified two LysM domain containing RLKs, LYK3 (or hcl; Catoira et al., 2001; T. Bisseling, personal communication) and LYK4 that are involved in IT formation (Limpens et al., 2003) (Table 1). The epidermal NF responses, such as multiple root hair outgrowths and swelling, but not curling, are still induced in the HCL mutant and can be uncoupled from infection and cortical cell division, because they are not induced. In this mutant, the expression of ENOD11 (Journet et al., 2001) and NIN (an early nodulin required for IT and nodule primordium formation; Schauser et al., 1999) is normal, favoring the model that LYK3 is an entry and not a signaling receptor. LYK3 and LYK4 are highly homologous to the LjNFR1 gene, but the loss-of-function phenotypes are different.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Legume species</th>
<th>Gene mutated</th>
<th>Reference</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>nfr5*</td>
<td>L. japonicus</td>
<td>LysM-RLK</td>
<td>Radutoiu et al. (2003)</td>
<td>Nod, attenuated and slower alkalization, but no other NF responses</td>
</tr>
<tr>
<td>hcl (=lyk3)***</td>
<td>M. truncatula</td>
<td>LysM-RLK</td>
<td>Endre et al. (2002) Stracke et al. (2002)</td>
<td>Nod/Myc, rh and reduced Ca²⁺ flux, no Ca²⁺ spiking or nodulin gene expression</td>
</tr>
<tr>
<td>symrk (RNAi)**</td>
<td>P. sativum</td>
<td>LRR-RLK</td>
<td>Lévy et al. (2004)</td>
<td>Nod/Myc*, normal Ca²⁺ flux and spiking</td>
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TABLE 1: Mutants in Nod factor perception

RLK, receptor-like kinase; LRR, leucine-rich repeat; CCaMK, calcium-calmodulin-dependent protein kinase; rhc, root hair curling; Nod, non-nodulating; Myc, no mycorrhizal association; IT, infection thread; NP, nodule primordium; LRB, lateral root base. * and ** indicate mutants in orthologous genes, and *** mutants that potentially encode orthologous genes.
NF binding has not been shown yet for any of these receptor molecules, whereas other proteins have been found with NF-binding specificity. For instance, NFBS1 and NFBS2 have been isolated from root extracts of M. truncatula and cell suspension cultures of M. varia Martym., respectively (Bono et al., 1995; Niebel et al., 1997) and NFBS3 has been associated to a high-density fraction of M. truncatula roots (Hogg et al., 2006). Interestingly, NFBS3 is absent in the mutants of the genes called DOES NOT MAKE INFECTIONS 1 and 2 (DMI1 and DMI2) (see below; Table 1) that are required for symbiosis with both rhizobia and mycorrhizal fungi, but is present in plants mutated in the NOD-FACTOR PERCEPTION (NFP) or DMI3 genes (Hogg et al., 2006). Another example of a NF-binding protein is a carbohydrate-binding lectin nucleotide phosphohydrolase from Dolichos biflorus (Lam.) Verdc. roots (Etzler et al., 1999).

Furthermore, several chitinase-like enzymes have been proposed to be involved in NF perception (Goormachtig et al., 2001; van der Holst et al., 2001). In S. rostrata, an acidic class III chitinase-like gene, Srchi24, is upregulated during LRB nodulation (Goormachtig et al., 1995, 2001) and its transcript levels increase 4 h after stem-located adventitious root primordia have been inoculated with NF-producing rhizobia. Srchi24 transcripts (Figure 3G) and proteins are located in the outer cortical cell layers and in the cells surrounding the IPs of developing nodules; the expression drops 7 days post inoculation (dpi), when nodules reach maturity. Srchi24 lacks an important catalytic glutamic acid residue that is replaced by a lysine residue, resulting in a loss of hydrolytic activity (Goormachtig et al., 2001).

Interestingly, a chitinase-related receptor-like kinase (CHRK1) located in the plasma membrane and isolated from tobacco (Nicotiana tabacum L.) also lacks a glutamic acid residue required for chitinase activity, resulting in the inability to degrade chitooligosaccharides or chitin (Kim et al., 2000). CHRK1-suppressed transgenic tobacco plants exhibited shotty callus growth, mimicking genetic tumors that are spontaneously formed on hybrids of tobacco (Lee et al., 2004). Thus, CHRK1-mediated signal transduction might play a role in tumor formation. The lack of hydrolytic activity has been observed in other chitinase-like proteins, such as concavalin B, a seed protein from Canavalia ensiformis (L.) DC (Schlesier et al., 1996), gp-39 secreted glycoprotein from human (Hakala et al., 1993), and another Drosophila melanogaster protein that might be a new growth factor (Kawamura et al., 1999). Srchi24 orthologs with a mutation in the catalytic center are not found by in silico hybridization of gene sequences from other legume species, such as M. truncatula, L. japonicus, or Glycine max (L.) Merr. (soybean). The chitinase-like RLK of tobacco and Srchi24 could be involved in perception of chitin-like molecules. A developmental role for signal molecules with a NF-like structure has been suggested in tobacco (Schmidt et al., 1993), carrot (Daucus carota L.) (De Jong et al.,
Chitinases or other NF-degrading enzymes can hydrolyze the NF chitin backbone to regulate NF amounts in the host. Plant chitinases are components of plant defense reactions, have antifungal activity, play a role in carrot somatic embryo development, and are involved in plant development (Collinge et al., 1993; Brunner et al., 1998). Based on various observations, a role during nodule development has been proposed for chitinases that cleave the β-1,4 linkages of the NF chitooligosaccharide backbone. Several chitinase isoforms are induced by rhizobia in the cortex of soybean nodules, probably to protect the central tissue against pathogen invasion (Staehelin et al., 1992; Xie et al., 1999b). In the M. sativa-Sinorhizobium meliloti symbiosis, chitinase expression in necrotic cells of the cortex might play a role in abortion of ITs (Vasse et al., 1993). In M. truncatula, various chitinases are induced during nodulation (Salzer et al., 2000). However, the NF structure with its specific modifications might protect against degradation and specific NF-degrading enzymes might be produced in certain hosts (Staehelin et al., 1994, 2000; for an overview, see D’Haeze and Holsters, 2002). NF degradation might be important for fine-tuning the regulation of NF amounts, avoiding continuous stimulation of NF perception that would elicit defense-like reactions (Savouré et al., 1997).

Chitinases and NF-degrading activities have also been induced during S. rostrata nodulation (Goormachtig et al., 1998a; D’Haeze and Holsters 2002). The Srchi13 gene, encoding an acidic class III chitinase (with 76% similarity to Srchi24) is induced in a NF-dependent manner. By in situ hybridization experiments, no signals for Srchi13 transcripts could be detected in uninoculated roots, whereas from 1 dpi on, expression was low in the cortex of the root primordium, with a maximum at 5 dpi, around IPs and ITs, in the nodule parenchyma, and in uninfected cells of the central tissue. The expression pattern together with the protein's capacity to degrade chitin and NFs imply a role in restricting NF responses (Goormachtig et al., 1995, 1998a).
To unravel signals that act downstream of NFs in LRB invasion, an axillary root hair assay has been used (Mergaert et al., 1993). Submerged roots of S. rostrata carry bulge-like structures at the junctions of primary and lateral roots. When $10^{-9}$ M of NFs is applied, these bulges elongate and are deformed, resulting in bushes of deformed axillary root hairs (Ndoye et al., 1994; Mergaert et al., 1993; D’Haeze et al., 2003). Axillary root hair formation is a valid and specific assay for NF responses during LRB invasion, but not for RHC invasion and can be mimicked by treatment with exogenous ethylene, H$_2$O$_2$, or gibberellin (GA). The obtained root hairs are mostly straight, in contrast to those induced by NFs. Inhibitors of ethylene synthesis or perception, scavengers or inhibitors of ROS, and inhibitors of GA synthesis block the NF-induced emergence of root hairs. Microscopic observation upon pharmacological treatments has revealed that ethylene and H$_2$O$_2$ act in the same pathway for bacterial invasion (D’Haeze et al., 2003). Moreover, inhibitors of ethylene, H$_2$O$_2$, or GA perception or synthesis block IP formation, indicating that these signaling molecules are necessary for IP formation during LRB invasion (D’Haeze et al., 2003; Lievens et al., 2005).

During S. rostrata nodulation, IP formation involves the induction of local cell death that coincides with a massive production of H$_2$O$_2$ and is required for deeper invasion of the host tissues (D’Haeze et al., 2003). ROS is also generated during nodulation of M. sativa: H$_2$O$_2$ has been localized in walls of infected cells and some ITs and O$_2^-$ has been found as well (Santos et al., 2001). ROS has been suggested to play a role in cross-linking of glycoproteins in the matrix of ITs of pea (Wisniewski et al., 2000) or in the induction of some nodulin genes, such as the peroxidase gene Rip1 in M. truncatula (Ramu et al., 2002). Also in the ITs of S. rostrata, James et al. (1996a) have detected glycoproteins that contain the same epitopes as those present in the IT matrix of pea nodules. In S. rostrata, bacteria in the IPs and in some ITs are enclosed in a matrix with high amounts of H$_2$O$_2$ (D’Haeze et al., 1998).

In wild-type A. caulinodans, exopolysaccharides (EPS) form a diffusion barrier that prevents the incorporation of H$_2$O$_2$ inside the bacteria, hence protecting them against H$_2$O$_2$ toxicity (D’Haeze et al., 2004), as shown through the mutants ORS571-X15 and ORS571-oac2. Both mutants produce normal amounts of NFs but have an altered lipopolysaccharides (LPS) O-antigen structure. Moreover, the ORS571-X15 mutant no longer produces EPS and is arrested in superficially located IPs upon inoculation of S. rostrata. No ITs are formed, indicating the need for a correct outer surface to enable the invasion of the plant tissue.
(D'Haeze et al., 2004). Upon inoculation of S. rostrata with the ORS571-oac2 bacteria that produce a thin layer of EPS in comparison to wild-type bacteria, IPs and ITs are formed, bacterial release occurs, but plant cells are never completely filled with symbiosomes. Because of the inefficient rhizobial invasion, nodule development is greatly retarded, resulting in multilobed non-functional nodules that are the consequence of a continued infection and primordium formation, without functional central tissue development (Mathis et al., 2005).

In summary, in addition to NFs, also EPS and LPS of A. caulinodans bacteria are involved in the nodulation process of S. rostrata. EPS protect the bacteria during the early stage of invasion against H$_2$O$_2$ that is required for LRB invasion. This conclusion is supported by the correlation between increased EPS production and enhanced protection against H$_2$O$_2$ in ex planta assays (D'Haeze et al., 2004). On the other hand, LPS are necessary as positive signals toward the plant cells for nodule progression and maintenance of a symbiotic interaction, repression of the pathogen defense plant responses, and insurance of bacteroid proliferation (Mathis et al., 2005).

The oxidative burst response of the plant upon bacterial invasion is consistent with the activation of the plant defense system, including the catalytic reactions of antioxidant enzymes. Secreted plant peroxidases are capable of either producing or scavenging reactive oxygen molecules. In M. truncatula, the Rip1 peroxidase gene is transiently induced by NFs upon S. melliloti infection (Cook et al., 1995). During RHC and LRB invasion of S. rostrata, the peroxidase gene Srprx1 is transiently upregulated in a NF-dependent manner, more specifically in cells around IPs and in cells flanking the ITs. Srprx1 expression stops accompanying the invading bacteria just before they enter the central tissue. The Srprx1 protein is able to oxidize several substrates in the presence of H$_2$O$_2$. Because wounding and pathogen infection could not induce Srprx1 expression, a nodule-specific function for SrPrx1 during bacterial invasion of S. rostrata has been proposed (J. Den Herder, unpublished results).

Another gene correlated with IP formation codes for a putative leghemoglobin, Srlb6. Transcripts accumulate in cells surrounding IPs and ITs during the early infection stages of S. rostrata (Figure 3E) (Den Herder et al., submitted). Leghemoglobins function as late nodulins in oxygen delivery to bacteroids (Legocki and Verma 1980; Appleby 1984; Long 1989). However, Srlb6 transcripts have been detected at the onset of rhizobial invasion, well before the start of bacterial nitrogen fixation (Goormachtig et al., 1995). In vetch (Vicia sativa L.), a similar gene is present and is often used as a marker for NF-induced effects (Heidstra et al., 1997a). Plant leghemoglobins or hemoglobins have also been related to detoxification of NO or halophenolics (Hérouart et al., 2002; Chowdhury et al., 2003; Dordas et al., 2003a, 2003b;
Perazzolli et al., 2004). Hence, a stress-induced plant response could lead to the synthesis of NO-binding molecules, such as SrLb6, to protect the plant cells against ROS during LRB nodulation.

During initiation of LRB nodulation, another gene is upregulated that encodes a GA20-oxidase enzyme (Lievens et al., 2001, 2005). This class of enzymes acts at the final stage of GA biosynthesis by converting inactive GA forms into bioactive GAs (Hedden and Phillips 2000; Yamaguchi and Kamiya 2000). In situ hybridization with a gene-specific probe of SrGA20ox revealed a transient, invasion-related transcript accumulation around IPs and young ITs. SrGA20ox transcript accumulation depends on NFs, placing the SrGA20ox functioning downstream from the NF signaling. Pharmacological experiments established that different inhibitors of GA synthesis negatively influence formation of IPs and ITs (Lievens et al., 2005), suggesting that GAs control IT structures. The inhibitory effect could be partially complemented by exogenous GA. Because ethylene and GA could act synergistically to promote plant cell death responses, they might play a role together in IP formation. In contrast, exogenous GAs, just like ethylene, inhibits the initiation of RHC nodulation in S. rostrata (Goormachtig et al., 2004b; Lievens et al., 2005).

**SIGNALS AND GENES FOR IT GROWTH**

By coinoculating S. rostrata with ORS571-X15 and ORS571-V44, complementation nodules are formed occupied by the V44 bacteria that are deficient in NF synthesis (D’Haeze et al., 2003). Nodulation is delayed and results in cauliflower-shaped nodules that develop at a frequency lower than that of the wild type (D’Haeze et al., 2003). A detailed analysis of these nodules showed that the EPS-deficient ORS571-X15 bacteria were only superficially located at the IPs, providing the signaling center of distant NF responses and allowing the ORS571-V44 strain to enter the plant and fix nitrogen. This restoration of symbiosis, with the formation of Fix+ complementation nodules, indicates that local NF perception is not needed for bacterial release inside the plant cells. On the other hand, proper IT formation requires local NF perception, because coinoculation results in irregularly shaped thicker ITs that grow with difficulty. Thus, local NF perception is probably necessary to fine tune the process and synchronize rhizobial invasion and primordium formation (Den Herder et al., submitted).

Additionally, plant defense-related gene expression, such as the induction of a pectin methyl esterase gene (SrPme1), a proteinase inhibitor gene (SrPI), and a chitinase gene (Srchi13), is observed around the ITs in the infection center. The overall expression pattern is
associated with uninfected cells (in the infection center, the nodule parenchyma, the infection zone, and the uninfected cells of the fixation zone) (Goormachtig et al., 1998a; Lievens et al., 2002, 2004) that might prepare defense responses as a protection against the rhizobia present in the neighboring cells. Pectin methyl esterases are involved in the demethylation of esterified pectin (a cell wall component) and could affect cell wall assembly; proteinase inhibitors prevent plant proteinase activity; and, chitinases have been shown to degrade bacterial peptidoglycan (Collinge et al., 1993). Furthermore, the Srpme1 expression is strongly associated with the new vascular strands (connecting vascular tissue) of the nodule (Figure 3I). Pectin methyl esterases modify pectin in the middle lamella of the cell wall and could be involved in xylogenesis of the vascular tissue (Fukuda 1996; Israelsson et al., 2003). This protein might determine cell fate by altering the properties of the wall or by facilitating the elicitor formation (Lievens et al., 2004).

Another gene that potentially modifies plant cell walls has been isolated by suppression subtractive hybridization of stem nodules of S. rostrata and encodes an S-adenosyl methionine synthetase (SrSAMS). In situ hybridization revealed expression in cells containing ITs and in some cells ready for IT passage (Figures 2I and 2K) (Schroeyers et al., 2004). Because S-adenosyl methionine synthetases are involved in ethylene synthesis, ethylene might play a role during IT progression.

**Signals and Genes for Primordium Formation**

The initiation of an NP is tightly regulated by balanced hormone concentrations and requires the induction of cell cycle genes, of which three have been isolated from S. rostrata stem nodules: a gene encoding a cyclin-dependent kinase 2 (Srcdc2), a cyclin B1 (SrcycB1:1), and a histone 4 (SrH4-1) (Goormachtig et al., 1997). In situ hybridizations revealed that Srcdc2-1 transcripts are abundantly present in all cells of the adventitious root primordia and developing nodules. A specific nodulation-related expression pattern has been observed for SrcycB1:1 and SrH4-1. Transcripts have been detected already at 1 dpi in the cortex of the adventitious root, reflecting reactivation of cell division with the formation of the NP as a result (Goormachtig et al., 1997). By analyzing the expression patterns of these genes, the indeterminate character of the developing nodules has been defined (see above; Goormachtig et al., 1997).

Various other genes, including ENOD40 and a RING finger protein-encoding gene, are expressed in the incipient NP (Corich et al., 1998; Compaan et al., 2001; Schroeyers et al., 2004). The SrRING transcripts disappear by the time the proximal-distal differentiation
gradient is established (Schroeyers et al., 2004), whereas SrENOD40-1 transcripts are present throughout the successive steps of nodule development (Figure 3F) (Corich et al., 1998). RING finger proteins provide regulatory functions in many signaling pathways, such as regulation of transcription factor expression, protein-protein interactions, or ubiquitin-dependent proteasomal degradation. Induction of SrENOD40 is enhanced as early as 4 h after bacterial inoculation (Corich et al., 1998). The nodule-associated expression is first seen in the root pericycle, opposite the protoxylem poles and in the incipient NP. Later on, SrENOD40 transcripts are only found back in meristem descendants that differentiate into the cells of the nodule central tissue or nodule parenchyma (Corich et al., 1998). It is still unclear whether ENOD40 functions as a protein or as a small RNA. The gene contains two conserved regions that might encode small open reading frames (Sousa et al., 2001). On the other hand, in M. truncatula, an RNA-binding protein (MtRBP1) has been identified that interacts with the full-length ENOD40 mRNA, relocating MtRBP1 from the nucleus to the cytoplasm. MtRBP1 accumulation in cytoplasmic granules has solely been observed in association with ENOD40 RNA (Campalans et al., 2004). How ENOD40 functions exactly is still unknown, but its gene product might be involved in growth regulation and cell differentiation, probably controlled by phytohormone landscapes, such as those of GAs.

Another example of the hormonal control on protein production is the hydroxyproline-rich cell wall protein SrEnod2 that is induced in the roots by exogenous cytokinin (Dehio and de Bruijn, 1992). This induction occurs at the post-transcriptional level and requires protein synthesis and protein phosphorylation (Silver et al., 1996).

**SIGNALS AND GENES FOR MERISTEM MAINTENANCE**

SrGA20ox transcripts have also been observed in cells of the preinfection zone, i.e., in differentiating cells just beneath the nodule meristem. Therefore, GAs might be involved in regulating cell differentiation. Also, in the shoot apical meristem, leaf primordium development is controlled by GAs, whereas KNOX homeodomain proteins repress GA20ox expression to maintain the undifferentiated status of the meristem cells (Barley and Waites, 2002; Vogler and Kuhlemeier, 2003). Interestingly, a KNOX homolog has been found in M. truncatula nodule meristems (Koltai et al., 2001), demonstrating that nodule meristems might not only have traits of root meristems, but also bear some shoot-like traits.

Ethylene controls the meristematic activity of S. rostrata nodules resulting in nodules of the indeterminate type on well-aerated roots and of the determinate type hydroponic roots.
This phenotypic plasticity is mediated by ethylene, because indeterminate nodules can be obtained under aquatic conditions when ethylene blockers are added to the medium (Fernández-López et al., 1998). The ethylene abundance under hydroponic growth conditions not only inhibits the RHC invasion and root hair growth, but also promotes the cortical invasion pathway through its involvement in IP formation. Expression patterns of four recently characterized genes can be linked to the presumed accumulation of ethylene in the nodule (Schroeyers et al., 2004). These genes include a gene coding for an S-adenosyl methionine synthetase (SAMS), an 1-aminocyclopropane-1-carboxylate (ACC) synthase (R. Mathis and S. Lievens, unpublished results), an ACC oxidase (S. Lievens, unpublished results), and a possible ethylene-responsive element-binding factor 1 (ERF1). SAMS, ACC synthase, and ACC oxidase could be implicated in the first (methylation reaction), second, and third (last) steps of the ethylene biosynthesis, respectively, whereas ERF proteins might function as transcription factors in the ethylene signaling cascade. Functional analysis has to be performed to show the involvement of these enzymes in aquatic root nodulation.

**Signals and Genes for Bacterial Uptake**

Sesbania rostrata is an excellent tool to study downstream functions of symbiotic genes that, upon mutation, cause a nodulation arrest at the level of the epidermis because LRB nodulation skips the epidermal perception mechanisms. SrSymRK is the ortholog of LjSYM1RK (Stracke et al., 2002), MtDMI2, MsNORK (Endre et al., 2002), and PsSym19 and encodes a receptor kinase with an amino-terminal signal peptide, an extracellular domain containing three leucine-rich repeat motifs, a transmembrane segment, and an intracellular serine/threonine kinase domain (Table 1). In RHC invasion, mutants of the NORK genes are blocked at the level of the RHC, partially because of interference in a NF-independent touch-responsive mechanism (Esseling et al., 2004). The mutants also lack NF-induced Ca\(^{2+}\) spiking responses and nodulation-related gene expression (Oldroyd and Downie 2004; Geurts et al., 2005). In situ hybridization with SrSymRK in developing stem nodules showed expression in the infection zone, i.e. in cells that take up bacteria. LRB nodulation of S. rostrata roots with RNA interference (RNAi)-mediated knockdown of SrSymRK expression, was affected at the level of IT structure and bacterial internalization, but not at the level of IT formation and NP development (Capoen et al., 2005; Table 1). This observation implies that SrSymRK, which is not detectably transcribed in either root cortex or NPs, is not an essential component of the NF signaling complex for triggering IT formation or cell division. ITs in the RNAi lines of
SrSymRK had a lumpy appearance, suggestive of abnormal matrix and wall properties. Similar results have been obtained for a weak allele of DMI2 in M. truncatula: the mutant allowed rhizobial infection but formed bulbous ITs, almost without bacterial uptake and inefficient invasion as a consequence (Limpens et al., 2005).

The major conclusions are that (i) during LRB invasion in the cortex SrSymRK is involved in IT structure and in bacterial uptake for symbiosome formation to establish the fixation zone and (ii) downstream of the epidermis signaling, it might not be coupled to the NF signaling receptor complex, but rather to a NF entry receptor complex that controls IT structure. Following our hypothesis, SrSymRK is part of a molecular complex that is recruited to allow an endophytic lifestyle of bacteria and fungi, incorporating cell wall integrity control and targeted exocytosis as part of a mechanism used by pathogens to force entry and by plants to allow symbionts to come inside. The cell-autonomous internalization of bacteria to form the fixation zone does presumably not involve local NFs, because the ORS571-V44 non-NF-producing bacterial strain could be internalized normally (D’Haeze et al., 1998; Den Herder et al., submitted), but required SrSymRK to function both in S. rostrata and M. truncatula (Capoen et al., 2005; Limpens et al., 2005).

CONCLUSIONS

The versatile growth and nodulation capacities of S. rostrata offer several advantages or unique opportunities for studying certain aspects of nodulation. Because two types of invasion occur on the same plant, these processes can be compared for hormone involvement, gene expression patterns (transcriptomics), and aspects of signal perception and transduction at epidermal versus cortical levels. Moreover, it is very easy to obtain mixed bacterial colonization in IPs, allowing extracellular complementation between mutant strains and facilitating the signaling role of purified compounds of bacterial origin.

As a consequence of flooding, ethylene accumulates at the nodulation sites and interferes with RHC nodulation and with initiation of root hairs. Thus, water-tolerant legume nodulation has recruited an alternative way that circumvents the epidermis and depends on ethylene in a cortical invasion process resembling aerenchyma formation coupled to NF perception. This type of adaptation probably arose several times independently in numerous taxonomic legume groups that developed water tolerance (as diverse as Aeschynomene, Discolobium, Neptunia, and Mimosa). For instance, in Neptunia plena, either RHC or LRB nodulation occurs depending on the environmental conditions (Goormachtig et al., 2004a).
Thus, in *S. rostrata*, the dependency of LRB nodulation on ethylene reflects the hormonal influence on processes, such as root growth, root hair distribution, nodule meristem maintenance, and intercellular invasion at LRB (Goormachtig et al., 2004b, 2004b). These observations support the finding that ethylene functions as a primary signal to activate water-adapted growth responses in *Rumex palustris* Smith. and deepwater rice (*Oryza sativa* L.) (Lorbiecke and Sauter 1999; Voesenek et al., 2003a, 2003b). Moreover, GAs and ethylene act synergistically and play a dual role in *S. rostrata* nodulation: they inhibit infection of rhizobia via RHC and promote LRB intercellular invasion under waterlogged conditions (D’Haeze et al., 2003; Lievens et al., 2005). Moreover, ethylene determines the nodule type of *S. rostrata* and the developmental expression pattern of *SrGA20ox1* suggests a positive role of GAs in differentiation of meristem descendants, the latter being a feature that is presumably common to both RHC and LRB nodulations.

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This study consists of two main parts which both focus on the functional analysis of plant genes in two different legume species. The first part (Chapter 3 and 4) uses the water-tolerant legume S. rostrata to improve the knowledge on the early Nod factor signaling events during lateral root base invasion. More specifically, an in-depth analysis on the role of the chitinase-like protein, Srchi24, is performed (Chapter 3). Srchi24 gene expression is upregulated during early nodulation and the protein was localized in the proximity of the Nod factors, indicating a role for Srchi24 in Nod factor perception and/or as adaptor or scavenger protein through NF-binding. Such a function was investigated by defining the subcellular protein localization, by a biochemical approach and a phenotypical analysis of transgenic roots overexpressing or knocking down Srchi24. In chapter 4, a more fundamental challenge is handled, A. tumefaciens-mediated transformation of the recalcitrant S. rostrata plant, by applying the multi-auto-transformation (MAT) vector system. If transgenic S. rostrata plants can be obtained, the functional analysis of stem- and root nodulation on the same plant species would be feasible.

In the second part, nodulation is studied in the model system for indeterminate root nodulation, Medicago truncatula (Chapter 5, 6 and 7). Chapter 5 and 6 describe the involvement of SINA E3 ligase proteins and NAC1-like transcription factors in nodulation, respectively. The Arabidopsis thaliana SINAT5 gene regulates the number of lateral roots by ubiquitination of NAC1, which transcriptionally activates genes that function in lateral root development. Because of the fact that nodule and lateral root formation display common developmental and morphological features, the M. truncatula orthologues or homologous family members might have been recruited for nodulation. In chapter 5, the phenotype of transgenic M. truncatula plants ectopically expressing SINAT5 or a dominant negative mutant gene is analyzed and a yeast-two hybrid and gene expression analysis are performed to reveal the regulatory roles of M. truncatula SINA proteins in nodulation and to identify which classes of proteins are potential targets for ubiquitination during nodulation. On the other hand, a detailed expression analysis and functional characterization of 3 NAC1-like genes in transgenic M. truncatula roots might give evidence for a nodulation-related function of this class of transcription factors (Chapter 6).

Finally, a primary insight to define nodule-related functions for a relatively new class of signaling molecules, CLE peptides, is examined through in silico identification and qRT-PCR based expression analysis of CLE genes in M.truncatula (Chapter 7).
CHAPTER 3

Functional analysis of *Srchi24* in *Sesbania rostrata*
**COVER:** Dark-field microscopical imaging of sectionned S. rostrata lateral root base nodule 5 days post inoculation with Azorhizobium caulinodans ORS571 expressing the β-glucuronidase gene. Gus-stained bacteria are seen as pink dots in the peripheral region of the nodule at the sites of infection pocket formation.
INTRODUCTION

Legume-rhizobium symbiosis involves the formation of nodule organs to establish a niche where the bacteria carry out nitrogen fixation. During this beneficial interaction, a repression of the plant defense-response is crucial to allow mutuality. How do plants distinguish whether the invasion by a micro-organism is pathogenic or symbiotic? The criteria to attain compatibility generally rest in the initial signals, such as the secretion of specific bacterial Nod factor (NF) molecules, and in the recognition mechanisms determining the downstream actions in the organisms. Plants primarily perceive external compounds through receptor proteins, such as the NF LysM receptors (Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003), although also other proteins can directly interact with the non-plant molecules in the apoplast to hamper (during pathogenesis) or to amplify (during symbiosis) invasion of the plant.

Plant chitinases were originally characterized as participants in plant-pathogen interactions, and were supposed to mainly function in defense (Boller, 1988). Chitinases contribute to antifungal activity through the degradation of fungal chitin, and the proteins are localized around fungal hyphae in planta (Wubben et al., 1992; Benhamou et al., 1993). On the other hand, chitinase gene expression has also been detected in organs that are not harassed by pathogens. The corresponding chitinases might function in certain developmental programs (Benhamou and Asselin, 1989). One example came from a carrot temperature-sensitive mutant cell line that was rescued by exogenously applied endochitinase during embryogenesis (De Jong et al., 1993). Another example is the presence of endogenous lipochito-oligosaccharides (LCOs), which are substrates of plant chitinases, in embryogenic cultures of Norway spruce (Dyachok et al., 2002).

Plant chitinases are grouped into six classes according to their primary sequence and structural features (Collinge et al., 1993). Class III chitinases belong to the family 18 of glycosyl hydrolases and show homology with fungal chitinases (Hamel et al., 1997). Most class III chitinases are secreted to the apoplast (Graham and Sticklen, 1994) suggesting an interaction with extracellular compounds. Plant chitinases have a variety of substrates, of which chitin, a β-1,4-linked polymer of N-acetylglucosamine (GlcNac), is the most common. Bacterial NFs or LCOs are also substrates of the bulk of plant chitinases (Perret et al., 2000), and hydrolysis results in biologically inactive NF degradation products (Roche et al., 1991; Staehelin et al., 1992, 1995; Ovtsyna et al., 2000). Moreover, several chitinase isoforms are induced by rhizobia in the cortex of Glycine max nodules (Staehelin et al., 1992; Xie et al.,
1999b). In fact, the subtle ability of the plant for differentiation between pathogenic and symbiotic responses is reflected in the recruitment of rhizobial induced chitinases, which may function in a protective way, such as the Srchi13 (Goormachtig et al., 1998a), but which might also function as a trigger for developmental changes. In this study, the role of the Srchi24 chitinase gene was studied during lateral root base nodulation of Sesbania rostrata.

The water-tolerant legume S. rostrata Brem (higher Papilionoideae) is an annual (semi-) tropical legume that interacts with Azorhizobium caulinodans ORS571 (Dreyfus et al., 1988; Dreyfus and Dommergues, 1981). Sesbania rostrata occupies temporarily flooded habitats and is used as green manure for rice cultivation. Upon submergence, S. rostrata develops adventitious roots from adventitious root primordia along its stem and after inoculation with A. caulinodans, nodules are formed on those sites as well as on the roots (for an overview, see Chapter 2). Depending on the growth conditions, nodulation on the root occurs either via the lateral root base (LRB) or via the ‘crack entry’ mechanism, which is a typical trait of aquatic nodulation, or via the root hair curling (RHC) process (Goormachtig et al., 2004b).

A. caulinodans is exceptional among rhizobia because of its dual capacity for free-living and symbiotic nitrogen fixation. The bacteria recognize their host through the flavanones liquiritigenin and/or naringenin exuded in the rhizosphere by the S. rostrata root (Goethals et al., 1989; Messens et al., 1991). This leads to the induction of the bacterial nodulation (nod) genes, which code for proteins responsible for the synthesis and secretion of NFs. In A. caulinodans, nod genes are located on the chromosome and are organized in different operons. Three nod loci were isolated: a regulatory, which contains the constitutively expressed nodD gene (Goethals et al., 1990), and two flavonoid-inducible ones, which are regulated by NodD and contain the nodABC SUJZ noeCH (Mergaert et al., 1996) and the noK (Mergaert et al., 1997c) genes (Goormachtig et al. 1998). All nodulation genes, except nodD, are symbiotically regulated because only in the presence of the plant specific flavonoids the NodD protein binds the nod box sequences to activate transcription of downstream-located nod genes. The nodA, B, C genes encode enzymes functioning in the synthesis of the NF backbone structure. This backbone consists of chitooligosaccharide moieties in which the N-acetyl group is replaced by an N-acyl group at the non-reducing end (Figure 1). The nodI and nodJ genes are involved in the secretion of the NFs outside the bacteria. The other nod genes produced by A. caulinodans, nodSUZ, noK and noeCH, contribute to the addition of specific substitutions on the NF chitin backbone (Figure 1),
determining host-specificity. The variability in species-specific NF structures is reflected in the length of the LCO structure (3-6 GlcNacs), in the nature of the fatty acid and in the modifications added to the backbone. A. caulinodans NFs are mainly chitopentasaccharides, carrying a palmitic (C16:0), vaccenic (C18:1), or stearic (C18:0) N-acyl chain, with an N-methyl and a 6-O carbamoyl group at the non-reducing end (D’Haeze et al., 1999). The reducing end remains unsubstituted or bears D-arabinosyl or L-fucosyl on C3 and C6 respectively (Figure 1) (Mergaert et al., 1993, 1997; D’Haeze and Holsters 2002). Undecorated NF structures, caused by mutations in the nod genes, can still nodulate S. rostrata through LRB nodulation, although nodulation is delayed and is less efficient (D’Haeze et al., 2000; Goormachtig et al., 2004b). Specific NF modifications also function in a protective manner by increasing the stability against degradation by NF-degrading enzymes, such as chitinases (Staehelin et al., 1994, 2000; Schultze et al., 1998). Chitin- and NF-degrading activities have been shown to be induced during S. rostrata nodulation (Goormachtig et al. 1995; Goormachtig et al. 2001; D’Haeze and Holsters 2002).

A differential display experiment comparing the RNA pool of uninfected root primordia and young developing stem nodules of S. rostrata revealed a differential expression forSrchi24 (Goormachtig et al., 1995). A basal transcript level of Srchi24 was present in the uninfected root primordium, namely in the young peridermal cells, followed

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**FIGURE 1:** Azorhizobium caulinodans Nod Factor structure. GlcNac backbone and N-acyl chain are shown in black. Strain-specific modifications are shown in red. The Nod proteins mediating specific synthetic features are shown in pink.
by an upregulated transcription 4h after inoculation in a NF-dependent manner. A maximum expression was detected at 5 dpi, while transcripts dropped after 7dpi, when mature nodules were formed. In situ hybridization and immunolocalisation on stem nodules revealed expression in the outer cortical cellayers and in the cortical cells surrounding the infection pockets (Goormachtig et al., 2001). In roots, a higher basal transcript level compared to the levels detected in the dormant root primordium on the stem was seen, which was localized in the root vascular tissue and the lateral root primordia (S. Goormachtig, unpublished results). Moreover, the expression in root nodules was lower than in stem nodules, and was restricted to the outermost cortical cell layer in the neighborhood of the infection pockets. Furthermore, a low expression level was observed in other plant parts, namely in young seedlings, and to a lesser extent in flowers. Gene expression was upregulated upon cytokinin treatment of the roots (Goormachtig et al., 2001), which can be correlated to the induction during nodulation because cytokinin positively influences nodulation and the expression of early nodulins, such as enod2 (Dehio and de Bruijn, 1992; Silver et al., 1996; Fang and Hirsch, 1998). Srchi24 transcripts and proteins co-localize with Nod factor-producing rhizobia during nodulation, which suggests a potential interaction with NFs.

The full length cDNA of the single-copy Srchi24 gene (EMBL Y12706) shows 56% to 76% homology with class III chitinases and encodes an acidic class III chitinase-like protein. The amino acid sequence contains an N-terminal signal peptide for endoplasmic reticulum (ER) targeting; the C-terminus is extended with 35aa compared to other class III chitinases (Goormachtig et al., 2001) (Figure 2). A highly similar extension was detected in Srchi13 (Goormachtig et al., 1998a) and a soybean chitinase (Yeboah et al., 1998). The Srchi24 catalytic domain for chitinase activity has a mutation of an essential glutamic acid residue (Glu150) to a lysine residue (Goormachtig et al., 2001; Figure 2), and recombinant MBP-Srchi24 fusion protein showed neither hydrolytic activity nor NF-degradation (Goormachtig et al., 2001). Other non-hydrolytic proteins resembling family 18 glycosyl hydrolases (Van der Holst et al., 2001) include concanavalin B, a seed protein from Conavalia ensiformis (Schlesier et al., 1996), the gp-39 and DS47 secreted glycoproteins from human (Hakala et al., 1993) and from Drosophila (Kirkpatrick et al., 1995), respectively, and the IDGF3 growth factor protein isolated in Drosophila (Kawamura et al., 1999). Interestingly, a non-hydrolytic chitinase-related receptor-like kinase (CHRK1) located in the plasma membrane and isolated from tobacco also lacks a glutamic acid residue required for chitinase activity (Kim et al., 2000).

Moreover, the mutated catalytic domain of Srchi24 probably results in a chitin binding and potentially in a NF binding domain because several chitinase-like proteins with
the same mutation display chitin binding capacity (Iseli-Gamboni et al., 1998; Renkema et al., 1998; Bleau et al., 1999; Van der Holst et al., 2001). Indeed, crude protein extracts prepared from tobacco BY-2 cell suspensions overproducing the protein demonstrated binding of Srchi24 to a regenerated chitin column (W. Van de Velde, PhD). Hence, the presence of Srchi24 in the proximity of NFs would allow the protein to bind the bacterial signal molecules without subsequent degradation, which would protect the NFs against active chitinases. As such, Srchi24 could function in prevention of NF degradation or as an adaptor protein in mediating NF perception through interaction with both NFs and NF receptor(s). This hypothesis was supported by the characterization of various NF binding proteins in other legumes via a biochemical approach. The Nod factor binding site 1 (NFBS1), isolated from M. truncatula roots, showed moderate affinity for the compatible S. meliloti NFs (Bono et al., 1995), although identification of a similar site in the non-legume tomato (Lycopersicon esculentum) suggested that this protein does not exclusively function in rhizobial symbiosis. The NFBS2 was characterized in microsomal fractions of Medicago varia cell suspensions, showing high binding affinity and selectivity for specific NF structural features (Niebel et al., 1997). The high affinity NFBS3 was detected in high density fractions of M. truncatula roots, is DMI2-dependent, and is believed to play a symbiotic role via NF binding (Gressent et al., 1999; Hogg et al., 2006).

![Alignment of amino acid sequences of Srchi24 and other chitinase-like proteins](image)

**FIGURE 2:** The amino acid sequences of Srchi24 (accession number Y12706) aligned with other chitinase or chitinase-like proteins including Srchi13 (Z48674), the Glycine max chitinase homologue (AB000097) and a similar M. truncatula protein encoded by the ORF in EST BG582391 (not full length, the N-terminal side end is unknown). Black and grey shaded amino acids are identical and similar, respectively, for 60% of 4 sequences. The conserved residues of the catalytic centre are labeled by *. The lysine residue replacing the glutamic acid in Srchi24 and MEST catalytic centers are labeled in red. The homologous CTE of all protein sequences, except the MEST protein, are boxed in green. The arrow indicates the potential cleavage site of the N-terminal signal peptide.
Furthermore, the NF-binding protein DB46 is differentially distributed along the surface of the Dolichos biflorus root axis in a pattern correlating with the zone of nodulation. Besides its presence on the surface of developing root hairs and its redistribution to the root hair tip upon treatment with NFs, it is also expressed in the root pericycle. The protein shows lectin-nucleotide phosphohydrolase (LNP) activity and is proposed to play a role in symbiosis or in related carbohydrate recognition (Etzler et al., 1999; Kalsi and Etzler, 2000). Lectin proteins, which confer carbohydrate-binding affinity, were characterized in different legumes (Hervé et al., 1996; Hirsch, 1999) and function during legume-Rhizobium symbiosis. For instance, lectins identified on the surface of roots promote aggregation of rhizobia (Diaz et al., 1995), which could be achieved through binding with rhizobial cell surface polysaccharides (Niehaus and Becker, 1998). The lectin-like receptor kinases (LecRK) characterized to date in M. truncatula among others, are predominantly expressed in roots. These proteins display a poor conservation in the residues involved in monosaccharide binding and are therefore proposed to function in the recognition of small hydrophobic molecules, such as complex glycans (Barre et al., 2002); alternatively, they might play a role in nodulation through heterologous oligomerization with soluble lectins (Navarro-Gochicoa et al., 2003).

In this advanced study on Srchi24, several approaches were applied to investigate whether this protein has a function in NF perception and/or NF binding. First, it was important to define the subcellular localization of the native Srchi24 protein, whereas NF binding is solely possible when the protein is present in the proximity of the NFs. Second, a biochemical approach was performed to demonstrate NF binding of Srchi24. The hydrolytic activity and the capacity for chitin- and NF-binding were analyzed in planta, but also purification of the native protein was undertaken. Third, a functional analysis involving the modification of Srchi24 expression levels in transgenic S. rostrata roots might reveal a role during LRB nodulation. Functional analysis was performed by a ‘reverse genetics’ approach, through the use of transgenic roots in which the expression of the gene is specifically modulated. In plants, this can be obtained by introducing overexpression or silencing constructs through Agrobacterium-mediated transformation (Wang and Wagner, 2003; Klahre et al., 2002). Silencing is obtained by introduction of sense (cosuppression), antisense or self-complementary RNA-duplex-forming (dsRNA or RNAi) constructs which results in induction of the plant’s post-transcriptional gene silencing (PTGS) mechanisms, acting through a sequence-specific degradation of transcribed RNA (Waterhouse et al., 1998). The natural way for induction of PTGS in plants, resembling RNA interference (RNAi) discovered in Caenorhabditis elegans (Fire et al., 1998), occurs as a response to plant RNA
viruses (Mourrain et al., 2000). PTGS and RNAi are mediated by small 21-25 nucleotide interfering RNA (siRNA) fragments, derived from double stranded RNA sequences degraded by the DICER RNAse, as sequence-specific targeting tools for the induction of silencing (Wang and Waterhouse, 2002; Elbashir et al., 2001; Bernstein et al., 2001). The introduction of dsRNA in which an intron is used as a spacer between the two RNAs shows the highest efficiency for PTGS induction in plants (Smith et al., 2000). Therefore, we used a sense construct to overexpress the Srchi24 gene and a dsRNAi constructs containing an intron sequence for downregulation of the transcript and protein level.

Finally, the basal expression level of Srchi24 in roots might indicate a function in (lateral) root development. Srchi24 gene expression alteration might influence root development and consequently hinder the nodulation capacity of these roots. Therefore, several inducible promoter systems were examined in S. rostrata.

RESULTS AND DISCUSSION

Subcellular localization of Srchi24
NFs are mainly located in the cell wall of the root hair and other epidermal cells, whereas at high concentrations, plasma membrane localization was also observed (Philip-Hollingsworth et al., 1997; Goedhart et al., 2003). Hence, proteins perceiving and transducing the bacterial signal inside the plant cells should be secreted or localized in the cell wall or plasma membrane. The Srchi24 protein sequence contains an N-terminal signal peptide for secretion. A C-terminal extension (CTE) of 35aa potentially includes an unknown localization signal. The same CTE is present in Srchi13; hence both proteins might show the same subcellular localization. Consequently, localization of both full length proteins as well as the truncated proteins, lacking the CTE, was performed.

Previous immunolocalisation of the Srchi24 protein in S. rostrata demonstrated its presence in the periphery of outer cortical cells of root primordia and stem nodules and around infection pockets of stem nodules, which all form potential sites for NF binding or perception. Electron-microscopy was carried out to distinguish between apoplastic or membrane localization but the antibodies were not sensitive enough. Other protein localization efforts were performed by western analysis using a polyclonal rabbit antibody raised against the maltose-binding-protein (MBP) Srchi24 recombinant fusion protein on protein extracts enriched from the medium of tobacco BY-2 cell suspensions overexpressing
Srchi24. This resulted in a faint band for Srchi24, indicating secretion from the tobacco cells (W. Van de Velde, PhD).

The cell wall, microsomal and cytosolic fractions of protein extracts of S. rostrata root and stem nodules 5 days post inoculation were separated by ultracentrifugation and analyzed by protein gel blotting in order to define the Srchi24 subcellular localisation. Antiserum specific for NtSyr, a conserved plasmamembrane localized Nicotiana tabacum syntaxin-related protein of 34kD, was used as a control (Leyman et al., 2000). NtSyr antiserum solely detected NtSyr in the microsomal fraction, confirming that fractionation happened correctly (Figure 3). Srchi24 proteins were present in all fractions including cell debris, cytosolic, microsomal and total proteins, which were prepared in the presence or absence of the denaturing agent dithiothreitol (Figure 3). Because the Srchi24 signal is present in the cell wall fraction of protein extracts from nodules at 5 dpi, we assume that the protein follows the secretory pathway and is localized in the apoplast. Its presence in the soluble and microsomal fraction is probably due to detection of the proteins during transport to the cell wall. The same result was obtained for Srchi13.

![Figure 3: Protein gel blotting on fractionated protein extracts of S. rostrata 5dpi stem nodules. Western analysis using MBP-Srchi24 polyclonal antibodies (upper) and Ntsyr antiserum (below) on 100μg total protein extract of the different fractions. Lane 1 and 5, celdebris; 2 and 6, total protein; 3 and 7, cytosolic fraction; 4 and 8: microsomal fraction. Lane 1-4, with 2.5mM DTT; lane 5-8, without DTT.](image)

Another approach to unravel the protein location involves transient expression of enhanced green fluorescent protein (eGFP, Clontech™, Haseloff et al., 1999) fusion constructs with Srchi24 and Srchi13 in epidermal cells of onion (Allium cepa) (Scott et al., 1999). The potential localization role of the CTE could be examined by targeting signal peptide-eGFP-CTE fusion constructs and truncated...
Srchi24 and Srchi13 GFP translational fusion proteins that lack the CTE. The different fusion constructs, listed in Figure 4, were made by ‘SOEing’ or overlap PCR (Horton, 1997) and introduced by site-specific recombination in the binary Gateway overexpression vector pK2GW7 (Karimi et al., 2002). After electroporation in A. tumefaciens LBA4404 and vacuum-infiltration in onion epidermal cells, the infected tissue was incubated at pH7 for optimal egfp fluorescence (Scott et al., 1999) (see Methods). Transient expression was analyzed by fluorescence and confocal microscopy (Figure 5).

The negligible autofluorescence observed in wild type onion epidermal cells (Figure 5B) allowed the localization of the egfp fluorescence in the different samples. Figure 5C shows that naked egfp, a small protein that is able to diffuse from cytosol to the nucleus, is present in these cellular locations, and when plasmolysis occurred (Figure 5D) the signal remained in the cytosol. The signal peptide sequence of Srchi24 (Figure 5E) and Srchi13...
(data not shown) fused to eGFP resulted in fluorescent signal in the cell wall. When the CTE sequence of Srchi24 and Srchi13 was fused to this SS-egfp construct (Figure 5) the same apoplastic GFP signal was detected (Figure 5F and G), indicating that this extension does not provide additional protein targeting signals. Plasmolysis of the cells displayed some remaining expression in the plasma membrane (Figure 5F), but this was a minor fraction compared to the egfp signal in the cell wall. Because of technical problems, the fusion with the full length sequence of Srchi24 was not available. However, the full length fusion construct was cloned for Srchi13 and for Srchi13 without CTE (Figure 4). Transient expression of both fusion proteins (with and without CTE) showed localization in the cell wall (Figure 5H, I).

In conclusion, it was shown that the N-terminal signal peptides of Srchi13 and Srchi24 target the proteins for secretion. The C-terminal extension, which is 70% homologous for Srchi13 and Srchi24 (Figure 2), does not function in the localization of the proteins. The full length Srchi13 protein is secreted, and because of the fact that Srchi24 shows high sequence similarity and no additional localization signals, also Srchi24 is most likely targeted to the cell wall. To summarize, we provide evidence that Srchi24 is primarily secreted to the cell wall, which does not exclude a potential interaction with NFs and a function in NF perception or protection.

**Activity of the Srchi24 protein produced in BY-2 cell suspensions**

Because Srchi24 is secreted from the plant cells, its potential NF-binding capacity was investigated using enriched protein extracts of transgenic tobacco (Nicotiana tabacum) Bright Yellow-2 (BY-2) cell suspension cultures (Nagata et al., 1992) that overproduced the Srchi24 protein. Also truncated (without CTE) and mutated (reversion of the Lys to Glu 150 amino acid in the catalytic centre) Srchi24 constructs were designed for overproduction in BY-2 cells. It was shown by W. Van de Velde (PhD) that in planta produced Srchi24 protein binds to a regenerated chitin column. This had to be confirmed using the newly transformed Srchi24-overproducing BY-2 lines. Furthermore, the hypothesis that the CTE, for which a helical structure has been predicted (Goormachtig et al., 2001), functions in chitin/NF binding is based on the fact that Srchi13, which encloses a homologous CTE, also binds to chitin despite its active non-mutated catalytic centre, and on the observation that a human chitinase belonging to the family 18 of glycosyl hydrolases binds to a regenerated chitin column via a C-terminal domain of 49aa (Tjoelker et al., 2000). So the chitin/NF binding capacity of truncated Srchi24 and Srchi13 (without CTE) was analysed in enriched protein
Functional analysis of Srchi24 in S. rostrata

FIGURE 6: Western blot analysis of transgenic BY-2 cell suspension protein extracts with polyclonal anti-MBP-Srchi24 antibody (A, B, and D) or anti-MBP-Srchi13 (C and D). A: lane 1, wt BY-2 control; lane 2 to 5, Srchi24 overexpressing lines. B: lane 1 to 4: Srchi24(Lys→Glu) overexpressing lines, lane 5 to 8: Srchi24(Lys→Glu)-CTE overexpressing lines. C: Srchi13 overexpressing lines in lane 1 to 4, Srchi13-CTE line in lane 5, and a wt BY-2 control line in lane 6. D: Chitin binding of Srchi13 and Srchi24 as shown by the different fractions eluted after loading the protein extracts on a regenerated chitin column. T, total protein extract; R, run-through fraction; w1 and w2, subsequent wash fraction; E, elution of the bound proteins in acidic conditions.

fractions from transformed BY-2 lines. Moreover, the likeliness that the mutation in the catalytic centre of Srchi24 increases the binding capacity of this chitinase-like protein was also tested with enriched protein fractions of BY-2 lines overproducing the reverted Srchi24 mutant protein. The fact that both, Srchi24 and Srchi13, bind chitin (W. Van de Velde, PhD) could be due to the presence of the CTE, but the binding for Srchi24 might be stronger because of the altered catalytic centre, thereby assuring that Srchi24 can scavenge or protect the NFs during nodule initiation.

BY-2 lines overexpressing Srchi24 or Srchi13 were selected for a high expression of the Srchi24 or Srchi13 proteins, respectively (Figure 6) (see Methods). Western analysis with polyclonal antibodies raised against MBP-Srchi24 and MBP-Srchi13 revealed that also the mutated and truncated proteins were detectable with this antibody (Figure 6). Crude protein fractions were loaded on a column packed with regenerated chitin and after washing off the unbound proteins, elution in acidic conditions was performed. Immunoblot analysis of the flow-through, wash and elution fractions revealed the presence of the Srchi24 and Srchi13 in the elution fraction, confirming chitin binding of both proteins (Figure 6). Unfortunately, when the lines overexpressing the truncated and mutated proteins were tested, the results were not conclusive. The truncated Srchi13 protein was only sometimes present in the elution fraction obtained with several experiments on the same line, and the immunoblotting analysis of the different fractions for detection of the mutated Srchi24 protein did not detect any eluted proteins. Furthermore, the polyclonal antibody for MBP-Srchi24 displayed far more unspecific binding affinity with other proteins present in the BY-2 protein extracts, resulting in western blots that did not allow a reliable analysis.
A protection-assay was carried out to get an indication whether the Srchi24 protein is capable of binding and protecting NFs against degradation by hydrolytic enzymes present in wild-type BY-2 cultures. The degradation pattern of a mixture of \(^{14}\text{C}\) labeled NFs and wild type BY-2 protein extracts was compared to the pattern obtained with a mixture of \(^{14}\text{C}\) labeled NFs and Srchi24 overexpressing BY-2 curde protein extracts. A set of different incubation conditions was tested: 0.1, 10 or 100 \(\mu\text{g}\) protein, incubated with NFs for 30 minutes, 2, 4, 8, or 18 hours either at 37° C or 0° C. Thin-layer-chromatography (TLC) analysis revealed that degradation of the labeled NFs already occurred after 30 minutes through wild type BY-2 hydrolytic proteins. Comparison with the degradation pattern of the transgenic 35S:Srchi24 BY-2 protein extracts incubated with labeled NFs did not result in a significant retardation or decrease in NF degradation (data not shown). Hence, the Srchi24 protein does not protect the NFs against active hydrolases, or the amount of active hydrolases in wild type BY-2 suspensions is much higher than the amount of Srchi24 produced, so that Srchi24 is not competitive with these enzymes to protect the NFs. Additionally, the specific activity of the \(^{14}\text{C}\) labeled NFs was way too low above background to observe minor differences in the degradation patterns. Another way to analyze the protective function of Srchi24 would be to purify the native protein and incubate this with \(^{14}\text{C}\) labeled NFs and a determined competitive concentration of active hydrolase enzymes, such as \((\text{GlcNAc})_4\) resin. The further analysis involving the NF binding capacity and hydrolytic activity potential of Srchi24 required the availability of pure Srchi24.

**Purification of the Srchi24 protein**

The overproduction of the different proteins in BY-2 turned out not to be a satisfying in planta system for massive protein production and subsequent purification. Hence, the production of Srchi24 proteins was carried out in the yeast Pichia pastoris. Yeast cultures overexpressing Srchi24 could facilitate purification of the native protein after secretion to the medium, which was shown to contain few other proteins (see results below). To be able to continue our biochemical analysis of the Srchi24 protein, cloning of the FLSrchi24, with and without CTE, and the reverted mutant Srchi24 gene in the pPICZC vector was performed and the constructs were transformed into Pichia pastoris cells (see Methods). Based on the previous observations (see above), secretion of the Srchi24 protein was expected. Seven different colonies containing the PICZC-Srchi24 plasmid were cultured in liquid medium, from which protein extracts were prepared and analyzed by western analysis with the
polyclonal antibody against MBP-Srchi24. However, no secretion of the protein was observed (data not shown), and this system was dropped.

On the other hand, in another approach the chitin-binding capacity of Srchi24 was used to purify the protein and subsequent testing biochemical characteristics, such as NF binding capacity, hydrolytic activity and protein interactions. A purification strategy based on protocols for purification of class III plant chitinases (Brunner et al., 1998; Yeboah et al., 1998; Kim et al., 1999; Van de Velde W., pers. com.) was set up for Srchi24 enriched protein fractions from overproducing BY-2 lines. Protein extracts were prepared from Srchi24-overproducing BY-2 cultures in Na-acetic acid buffer pH5 and loaded on a column packed with regenerated chitin to wash off proteins other then the chitin binding ones.

Further purification of these enriched protein fraction was required to obtain pure Srchi24 protein. Therefore, another approach was launched, namely the isolation of a monoclonal weak-affinity antibody specific for Srchi24, by the application of the Phage-Display Single Chain variable Fragment (ScFv) library (De Jaeger et al., 2002; Eeckhout et al., 2004). In general, the strong binding between an antibody and its epitope-containing protein is too strong to be reverted, but affinity-chromatography using a weak monoclonal antibody against the Srchi24 protein would allow us to elute the pure Srchi24 protein after specific binding. The Phage-display library was screened with purified recombinant MBP-Srchi24 protein, and after three rounds of panning, one candidate clone was detected for this antigen. Enzyme-linked immunosorbent assay (ELISA) revealed the antigen-binding affinity of the ScFv for the Srchi24 part of the folded recombinant MBP-Srchi24. Subsequently, the binding-affinity for the Srchi24 protein was analyzed. Therefore, periplasmic extracts of the ScFv fragment, which is coupled to a cMyc-tag, were prepared from E. coli and analyzed for binding to Srchi24. Protein extracts of Srchi24-overexpressing BY-2 lines and of wild type BY-2 lines were prepared in two different extraction buffers (see Methods). SDS-PAGE and subsequent immunoblotting was performed for detection of Srchi24 with polyclonal antibody against MBP-Srchi24 (as a control for protein expression) and with the periplasmic ScFv-containing extract and anti-cMYC antibody. However, no specific or differential signal was observed comparing transgenic and wild type BY-2 extracts.

Therefore, the ScFv was cloned in the PICZa(Fc) vector (see Methods) and transformed in Pichia pastoris GS115, resulting in the ScFv-Fc fusion (Figure 7A). Transformed Pichia cultures produce the bivalent ScFv-Fc antibody secreted in the growth
medium in which few other proteins were detected via silver staining of the proteins extracted (Figure 7B). This antibody production method allowed an easy purification of the antibody through NH₄SO₄ precipitation and protein A binding (see Methods). Immunoblotting using an anti-Fc human antibody for detection of the pure ScFv-Fc antibody showed that a desirable amount of protein was purified in this procedure (Figure 7B). ELISA analysis with the MBP-Srchi24 recombinant protein confirmed the antigen-binding affinity of the purified ScFv-Fc bivalent antibody. Analysis of the recognition capacity of the Srchi24 was performed via western blot using this purified antibody on protein extracts of Srchi24 overexpressing BY-2 cells, on wild type BY2 cells (as a negative control), and on MBP-Srchi24 pure protein. No differential signal could be detected for Srchi24 BY2 extracts compared to wild type BY-2 extracts, indicating that the Srchi24 protein is not recognized by the candidate antibody. Moreover, the denatured MBP-Srchi24 protein could not be detected either, implying that the antibody only recognizes a native epitope of the MBP-Srchi24 recombinant protein. Indeed, using the pure monoclonal antibody for immunoprecipitation of Srchi24 or wild type BY-2 crude extracts, and of purified recombinant MBP-Srchi24 supplemented wild type BY-2 protein extracts, a signal for MBP-Srchi24, but sadly not for Srchi24 was detected (Figure 7C). Thus, despite the fact that the epitope for the monoclonal antibody was specific for the Srchi24 part of the folded MBP-Srchi24 protein, binding affinity for the unfolded MBP-Srchi24 was abolished as for the Srchi24 protein.

![Image](image.jpg)

**FIGURE 7:** ScFv antibody production in Pichia pastoris. **A**, pPICZalfa(Fc) vector and antibody structure; **B**, Silver stained protein gel with proteins extracted from the growth medium of 4 P. pastoris pPICZalfa(ScFv-Fc) after 72 h of growth, showing that few other proteins are detected in the growth medium. The lower image is an immunoblot after purification of the antibody. The anti-human Fc antibody was used for detection of the ScFv-Fc antibody fragment. **C**, Western blotting with the polyclonal antibody against MBP-Srchi24 to detect the recognition capacity of the ScFv-Fc purified antibody for Srchi24 after immunoprecipitation. No differential band for the Srchi24 could be observed between the BY2-Srchi24 and BY2-wt extracts precipitated. The MBP-Srchi24 was shown to be immunoprecipitated (77kD).
The isolation of only one candidate ScFv for specific binding with Srchi24 was not a successful panning result. Therefore an additional screen of the Phage-display library was done with freshly purified MBP-Srchi24 (see Methods), but no new candidate clones were identified. In conclusion, the efforts to obtain a satisfying method for purification of the native Srchi24 partially succeeded, namely the in planta produced Srchi24 was enriched by binding to regenerated chitin. However, the subsequent step necessary for final purification by the isolation of a weak affinity monoclonal antibody did not succeed. Therefore, we decided to postpone the complex purification that could alternatively be performed by applying classical affinity-chromatography, and to focus on the functional analysis of Srchi24 in S. rostrata.

Functional analysis of Srchi24 in transgenic S. rostrata roots
A. rhizogenes transformation was applied to obtain transgenic S. rostrata roots (Van de Velde et al., 2003) containing an overexpression, silencing (dsRNAi) or control T-DNA, in all of which transcription was driven by a constitutive CaMV35S-promoter. The T-DNA region of the binary vectors also carried a gus or egfp reporter gene (pPZP200 derived constructs provided by W. Van de Velde) (Figure 8). The same type of constructs were made using the Gateway technology (Karimi et al., 2002; Invitrogen) to test whether we could obtain a higher efficiency of knock-down or overexpression, but comparable degrees were obtained with these plasmids.

![Diagram](image)

**FIGURE 8:** Srchi24 overexpression and silencing constructs.
Expression is driven by the constitutive 35S promoter and terminated at the 3'-ocs terminator sequence. In between these sequences, the full length Srchi24 sequence is inserted for overexpression and an RNAi construct is inserted for knock-down of Srchi24. The RNAi construct consists of the ORF of Srchi24 in sense orientation followed by the ORF in antisense orientation with between the two the cyclin-dependent-kinase (CDK) intron sequence, and transcription results in an intron-spliced hairpin dsRNA construct.

Because of the expression of Srchi24 detected in uninoculated roots, which indicates a role for Srchi24 in root development, a statistical analysis was performed on the transgenic
gus-positive roots formed after introducing RNAi, sense, and control constructs by A. rhizogenes transformation. Two independent experiments demonstrated that the population of roots transformed with the RNAi construct produced significantly less co-transformed hairy roots compared to the control population (W. Van de Velde, PhD and G. Den Herder), which might indicate that root formation is blocked during the transformation events where Srchi24 is effectively silenced. Thus, assuming that Srchi24 influences root development, nodulation can only be studied by using an inducible system (see below). However, phenotypical analysis was initiated with the hairy root lines in which the sense or RNAi construct was under control of a 35S constitutive promoter, and using an egfp marker allowing in vivo screening of cotransformed hairy roots.

A. rhizogenes infection of the different plasmids resulted in five transgenic hairy root lines containing the empty control vector, 11 lines with the overexpression construct and 21 lines carrying the RNAi constructs. Transgenic hairy roots were propagated in vitro (see Methods) to collect root material for determining the protein levels, while the chimeric plants were grown in hydroponic conditions and subsequently inoculated with A. caulinarodans ORS571 to analyze the nodule phenotype.

Western analysis on an equal amount of total protein extract with the polyclonal antibody against MBP-Srchi24 revealed the difference in protein levels. The control lines all showed a moderate comparable level of Srchi24 protein, whereas variable levels were observed in the knock-down lines. Six out of 21 RNAi lines displayed a reduced protein level, and in 3 lines no Srchi24 protein was detected, resulting in a knock-down effect in 9 lines (Figure 9). Only one out of 11 transgenic lines containing the overexpression construct displayed a higher amount of Srchi24 protein (Figure 9).

![Figure 9](image-url)
After inoculation with A. caulinaris, the control lines all nodulated normally, whereas the overexpression line did not develop any nodules. The hairy root culture overexpressing Srchi24 contaminated during in vitro growth, not allowing any further analysis. Several new A. rhizogenes infections were performed with a strain containing the overexpression construct or containing a newly designed overexpression construct by using the Gateway technology. However, the very low efficiency (1/11) to obtain an increased amount of Srchi24 protein was even more pronounced, because on 25 hairy root lines tested none of them showed an overproduction of Srchi24. In general, the overexpression mechanism works very efficient in plants (90 % efficiency for Srchi13 overexpression, data not shown), so it might be that Srchi24 expression is regulated on a post-transcriptional level, namely on the protein level.

Inoculation of the RNAi lines resulted in normal nodule formation. Even the lines with a total knock-down produced nodules. For a more detailed analysis of the nodulation process in these lines, one line with normal, 3 with decreased, and one with no Srchi24 protein production, transgenic roots were grafted (Figure 10) and inoculated to analyze nodule morphology. Grafting experiments were very inefficient but essential for further analysis of these lines from which the protein level had been determined. A decreased root growth rate and delayed nodule development were observed in all grafted plants. Nodules were counted and harvested to compare the morphological effects in different clonal hairy root lines by microscopical analysis at 5, 7 and 10 days after inoculation. Neither the amount of nodules nor the morphological analysis of the nodules on the roots with a reduced Srchi24 protein level revealed differences as compared to lines with a normal protein level (Figure 11). Also no abnormal localization of bacteria or aberrant nodule development was observed. From these results we concluded that Srchi24 could not have an essential function during S.rostrata LRB nodulation.

**FIGURE 10**: Grafts of transgenic hairy roots and wild type upper plant parts of 1 week old seedlings. Arrow: connection between root and green part after ten days of in vitro growth in the tube.
Inducible promoter systems in *S. rostrata*

To investigate the function of nodule-upregulated genes that are also expressed in other organs, a suitable system for inducible transgene expression was examined. Several inducible promoter-systems (reviewed by Padidam, 2003) were tested in *S. rostrata* transgenic roots, in which gus reporter gene expression is driven by the inducible promoter, allowing visualization of induction by blue staining.

First we explored the GVG inducible system, containing a Gal4 DNA binding domain, the Herpes simplex VP16 transactivation domain, and activation via a glucocorticoid receptor (GR) regulatory domain upon addition of dexamethasone (Figure 12A). We cloned a gus-intron marker gene in the MCS and the prolD-egfp expression cassette in the T-DNA region for in vivo screening of the transgenic roots. In the meantime, research from the group of Ian Moore (pers.comm.) showed that this system often shows patchy expression and that
frequently silencing occurs due to methylation of the Gal4 upstream-activated-sequence (UAS). Consequently, we switched to another system, the XVE inducible system (Zuo et al., 2000), with a Lex DNA binding domain, the VP16 transactivation domain, and the 17β-estradiol (2µM)-inducible estrogen regulatory (ER) domain (figure 12B). In S. rostrata, 88 transformed explants were tested in various conditions (see Methods), but either a background gus expression (blue staining without induction) was detected or no gus-staining upon induction could be obtained. Hence, this system has leaky promoter activity in S. rostrata transgenic roots grown in hydroponic conditions, which is possibly due to a high endogenous estrogen level in S. rostrata. Another legume, Lotus japonicus, did not show background activity, but had an induction-efficiency of only 2.4%, which is very low (Andersen, Denmark, pers. comm.). The third system tested in S. rostrata involved the pJCGLox system (Joubès et al., 2004), which is a ‘double-lock’ system based on the pJCrox system (Hoff et al., 2001), and combining the Cre-loxP site-specific recombination system and the subcellular targeting of proteins by the mammalian glucocorticoid receptor (GR) (Figure 12C). In BY-2 and Arabidopsis thaliana cell suspensions, the induction of the gene of interest was nicely correlated with the disappearance of egfp signal after excision of the egfp gene by the CRE recombinase, but in whole plants, no correlation between induction and excision could be made (L. De Veylder, pers. comm.). Finally, also this system did not allow controlled gene-induction in S. rostrata.

**FIGURE 12:** Inducible promoter constructs analyzed in S. rostrata. 
**A.** pIndex3 inducible GVG vector region. **B.** XVE inducible promoter construct. **C.** pJCGLox construct. MCS, multi-cloningsite; gus, gus marker gene with intron; GR, glucocorticoid regulatory domain which binds to GVG sequence upon dex induction; GVG, Gal4 DNA binding domain and VP16 transactivation domain with 35S region. HSP, heat shock promoter; Cre, CRE recombinase gene; intr, intron; GR, glucocorticoid regulatory domain; 35S, CaMV 35S promoter; T, terminator sequence; egfp, enhanced green fluorescent protein; Lox, Lox recombination site.
In silico identification of Srchi24 orthologues

The Srchi24 full length sequence was used as an in silico probe to identify potential orthologues in other legume or model plants. TBlastX analysis on EST databases of several species, including M. truncatula, Lotus japonicus, Glycine max, Phaseolus vulgaris, Oryza sativa, and Arabidopsis thaliana mainly resulted in the in silico isolation of chitinase genes displaying a conserved catalytic centre, which implements that only active chitinases and not chitinase-like proteins were detected. However, in the M. truncatula EST database, one EST (BG582391) (Figure 2) was identified in a cDNA library of 1 month old nodule tissue of which the gene product contains a glycosidase hydrolase class III acidic endochitinase domain that carries the same mutation in the catalytic centre as Srchi24. The 156 amino acid protein (which is not FL at the N-terminus) has 66 % similarity with Srchi24 and was predicted to have an N-terminal signal peptide (Figure 2).

However, the TC sequence to which this EST sequence belonged showed a predicted ORF encoding protein which contains a functional catalytic centre without mutations in the essential residues. No further interest in this sequence was retained because of the probability that sequencing mistakes occurred in this single EST and because of the lack of prove for a nodulation specific function for Srchi24 during LRB nodulation.

CONCLUSIONS

In this advanced study on Srchi24 function, the subcellular protein localization analysis revealed that the native protein is secreted to and present in the cell wall. Biochemical evidence for the lack of hydrolytic activity and the binding of Srchi24 to chitin provided further evidence for a possible interaction with NFs and its function in scavenging NF molecules for perception by the receptors. However, neither the binding with NFs nor a possible influence of Srchi24 to protect the NFs against active hydrolases was demonstrated in planta. Moreover, transgenic S. rostrata roots in which the Srchi24 protein is absent did not show any phenotypic defect in LRB nodulation. From these results we concluded that the Srchi24 gene is probably not essential during S. rostrata LRB nodulation or that the gene is functionally redundant. We are currently not aware of the existence of other S. rostrata chitinase-like proteins, but hardly any genomic data for S. rostrata are available. The identification of one putative chitinase-like protein in M. truncatula containing the same mutation in the catalytic centre indicates that this mutation is not as common, and might still involve a nodulation-specific feature. Another possibility is that Srchi24 cooperates with
other proteins in a complex to exert its function. Maybe a specific protein interaction is necessary for NF binding of Srchi24 by a modification of its structure. According to this hypothesis, overexpression of Srchi24 would not per se affect nodulation.

Further, a role in AM symbiosis is still a possibility, but was not investigated in this study.

In conclusion, no function could be assigned in the entrapment of NFs to protect against hydrolytic enzymes or to facilitate interaction with a receptor protein during S. rostrata nodulation. On the other hand, the weak basal expression of Srchi24 in uninoculated roots, in seedlings and flowers, the induction of Srchi24 by exogenous cytokinins, and the upregulation by NFs during S. rostrata nodule formation (Goormachtig et al., 2001) could still imply a function related to nodule organ development. A non-symbiotic role in organ development is further supported by the reduced root number formed upon RNAi of Srchi24.

**METHODS**

**S. rostrata Brem. plant material: seed sterilization, germination, and inoculation**

Sesbania rostrata Brem seeds were sterilized as described by Goethals et al. (1989) and germinated on Petri dishes containing 0.8% (w/v) agar in tap water in the dark at 28°C for 2 days. The seedlings were transferred to pots containing equal amounts of potting soil and sand. The plants were grown at 28°C with a 16h light period for 2-3 months. The root primordia on the stem were infected by painting with a bacterial inoculum, harvested by peeling off from the stem, and frozen in liquid nitrogen (Goormachtig et al., 1995). For the root nodulation, the plants were grown in tubes containing sterile nitrogen-free Norris medium (Vincent, 1970) and inoculated after 1 week as described by Fernández-López et al. (1998). Inoculation with A. caulinodans ORS571 wild type and A. caulinodans ORS571 (pRG960SD-32) (Van den Eede et al., 1992), carrying a β-glucuronidase (gus) marker gene, was performed as described (D’Haeze et al., 2000).

**A. rhizogenes transformation of S. rostrata**

This transformation method was performed as described in Van de Velde et al. (2003).

**Root culture propagation and grafting of S. rostrata transgenic roots**

Hairy roots were propagated in vitro by overnight incubation of root pieces (at least 1cm) in liquid medium, consisting of ½ MS, 1 % sucrose, 100 mg/L cefotaxime (Cf) and 500 mg/L
carbenicillin (Cb), at room temperature in the dark. The root pieces were transferred to Petri dishes with medium containing ½ MS, 2% sucrose, supplemented with 100 mg/L Cf and 300 mg/L Cb, grown at 22°C in the dark and transferred to new Petri dishes every 2 weeks.

In vitro cultured transgenic roots were grafted to the upper part of one week old S. rostrata seedlings. The bottom of the hypocotyl, which was gently cut while held in water, was connected to the top of the root piece by holding the two parts together in a sterilized plastic tube of 1 cm long and 1.5 mm in diameter. Subsequently, the grafts were grown in vitro on MS medium supplemented with 2% sucrose, 0.27% phytagel, Cf^{100} and Cb^{500} for one or two weeks, transferred to a hydroponic culture (falcon tube containing ½ Norris medium) for another week, followed by inoculation with A. caulinodans ORS571 (pRG960SD-32).

**Plant treatments**
Transgenic roots appearing around 1 month after A.rhizogenes infection were tested for induction of the XVE promoter in hydroponic conditions or in vitro on plates. A range of concentrations of 17β-estradiol (Sigma Aldrich) (0, 2, 2.5, 5, 10, 20 µM final concentration) was supplemented to the liquid or solid medium during a period of 8, 16, 24 or 32 hours.

Histochemical localization of β-glucuronidase activity in the transgenic roots was done as described previously (Van de Eede et al., 1992). The presence of blue cells/tissue was analyzed with a stereo microscope MZFLIII (Leica, Wetzlar, Germany).

**Visualization of EGFP fluorescence and confocal microscopy**
In vivo screening of transformed cells for EGFP expression was done as described by Van de Velde et al. (2003). Confocal microscopy was performed according to Vanstraelen et al. (2004).

**Protein extraction from S. rostrata hairy roots**
Hairy roots for immunoblot analysis were grinded in liquid nitrogen in a chilled mortar and the powder was resuspended in extraction buffer (50mM Tris-Cl pH 8.5, 10mM β-mercaptoethanol) in a 1:1 powder/buffer ratio and centrifuged at 10 000xg for 30 min at 4°C, thereby separating soluble proteins from the remainder. Protein concentration of the supernatant was determined by using the Biorad Protein Assay kit (Bio-rad, Hercules, CA) together with several concentrations of bovine serum albumine (BSA) as a standard. For immunoblot analysis, 50 µg of total protein was loaded on protein gel.
**Microsomal fractionation of S. rostrata protein extracts**

S. rostrata stem and root nodules were harvested 5dpi and frozen in liquid nitrogen. The material was grinded and protein extraction was performed in 1:1 (w/v) extraction buffer consisting of 300mM sucrose, 25mM Tris-HCl pH 8, 5mM EDTA, 15mM MgCl₂, 85mM NaCl, 0.1% Tween-20 and proteinase inhibitors (Complete™, Boehringer Mannheim, Germany), with or without 2mM dithiothreitol (DTT; New England Biolabs). Extracts were homogenized by vortexing and rotation at 4°C for 1h. After centrifugation at 4°C for 10 min at 10 000xg, the supernatant was transferred to a new tube and recentrifuged at 14 000xg for 20 min at 4°C, resulting in the pellet containing the cell debris and the supernatant containing the total protein extract. Ultracentrifugation at 100 000xg and 4°C for 1h resulted (Beckmann TLS55 rotor) in a second fractionation, the soluble cytosolic protein fraction and the pellet, consisting of the microsomal proteins. This pellet was washed for 3 times with extraction buffer and resuspended in 100 µl 10% SDS, 10% Triton-X 100, and the protein concentration of all fractions was determined by using the Biorad Protein Assay kit (Bio-rad) with BSA dilutions as a standard. For immunoblot analysis, 100 µg of total protein was loaded on protein gel.

**SDS-Polyacrylamide Protein gel electrophoresis (SDS-PAGE) and immunoblot analysis**

Protein extracts were dissolved in Tris-Glycine-SDS (TGS) sample buffer (Novex, San Diego, CA) supplemented with 100mM β-mercapto-ethanol, boiled for 5min at 95°C and loaded on protein gels. SDS-PAGE was performed according to Laemmli (1970) with 10 % polyacrylamide-resolving gels and 5 % (v/v) polyacrylamide-stacking gels. Electrophoresis was performed in 1x TGS running buffer (Biorad) at 120V. The separated proteins were transferred electrophoretically by wet blotting onto an Immobilon-p PVDF membrane (Millipore, Bedford, MA) at 55 V for 2h. Membranes were blocked overnight in 5 % skim milk suspended in TBS-T buffer (100mM Tris-Cl, pH 7.5; 150mM NaCl; 0.1% Tween-20) at 4°C and subsequently washed 3 times in 1x TBS-T for 10 minutes. Srchi24 and Srchi13 proteins were specifically detected by incubation of the membrane for 1h30 min at room temperature with a rabbit polyclonal antibody raised against the recombinant MBP-Srchi24 and MBP-Srchi13 fusion protein, respectively (antisera diluted 1/3000 in 5 % skim milk in TBS-T). For detection of the NtSyr proteins, a rabbit polyclonal antibody was used in a 1/2000 dilution in 5 % skim milk in TBS-T. After washing with TBS-T buffer, membranes were incubated for 1h.
at room temperature with an anti-rabbit IgG secondary antibody coupled to alkaline phosphatase (AP) (1/25 000) and revealed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (Biorad). Alternatively an IgG secondary antibody coupled to horse radish peroxidase (HRP) was used (1/10 000), which was detected with a chemiluminescence detection kit according to the manufacturers instructions (Perkin-Elmer Life Sciences, Inc., Boston, MA).

**Protein silver staining**

The protein gel was incubated for 30 minutes in a solution of 50 % methanol/ 10 % acetic acid and subsequently washed for 1 h in a solution of 5 % methanol/ 7 % acetic acid, followed by incubation for 1h in 10 % glutaraldehyde. The gel was extensively washed with water and then incubated for 15 minutes in the silver stain solution (per gel: 21ml 0.36 % NaOH, 1.4 ml NH₄OH, 0.332 g AgNO₃ and 75 ml water). After 3 washing steps with water, revealing was performed with reducing solution containing 0.5 ml formaldehyde (37 %) and 5 ml citric acid (1 %) in 1 L water. After visualization of the proteins, the reaction was arrested with 5 % acetic acid.

**Manufacturing constructs**

The ‘SOEing’ or overlap PCR fragments (Horton, 1997) were obtained by amplification with primers designed as in Figure 4. PCR of the separate fragments with an additional small amount of base pairs necessary for the sequence overlap were performed on the required template DNA’s (pBluescript-didi24, pBluescript-didi13 or pPZP200(35S-egfp-T) plasmid DNA). The final PCR reaction was performed with the obtained overlap PCR fragments as template and with primers containing the start or end sequence of the construct followed by the attB sequences for site-specific recombination in the pDONR201 entry vector and subsequent introduction in the destination vector pK2GW7 (Invitrogen, Karimi et al., 2002).

PCR fragments of FLSrchi24, FLSrchi13, FLSrchi24-CTE, FLSrchi13-CTE, FLSrchi24(Lys→Glu) and FLSrchi24(Lys→Glu)-CTE were obtained by amplification with gene-specific primers with in addition the attB recombination sites necessary for site-specific recombination in the pDONR201 or pDONR221 Gateway entry vector, which was subsequently recombined with the binary overexpression vector pK2GW7 (Invitrogen, Karimi et al., 2002). To obtain correct amplification products, all PCR reactions were performed using Pfx proofreading or High-fidelity (Hifi) Taq DNA polymerase (Invitrogen, Life technologies, USA).
Cloning of the FLsrchi24 fragment in the pPICZC vector for production of Srchi24 in P. pastoris GS115 was done as followed. The pPICZC plasmid was digested with SsnBI, and the FLsrchi24 was cut from the pDONR201(FLsrchi24) vector with ApaI and NruI enzymes, and cloned in pPICZC. The pPICZC(FLsrchi24) vector was subsequently transformed in E. coli and after plasmid DNA preparation, 10 µg of pPICZC(FLsrchi24) plasmid was linearized with PmeI and used for electroporation in P. pastoris.

For the production of the monoclonal ScFv-Fc antibody in Pichia pastoris, the ScFv sequence, encoding the single chain variable fragment (ScFv) specific for an epitope on MBP-Srchi24, was cut from E. coli HB2151(alfachit8) by SfiI and NotI and cloned in the yeast transformation vector pPICZalfa(Fc). This vector contains the human Fc (IgG1) fragment, a c-myc and his6-tag and was previously cloned as described in, and provided by, Eeckhout et al. (2004). The resulting pPICZalfa(ScFv-Fc) plasmid was used for heat shock transformation in E. coli MC1061 and incubated on low salt LB medium with 25 µg/ml zeocin for selection of the plasmid. Restriction digests allowed selecting the positive colonies. 3 µg of PmeI linearized plasmid was used for transformation of P. pastoris GS115.

Subcellular localization of GFP-fusion proteins in onion epidermal cells

Onion peels were cut in 1.5x1.5 cm pieces and incubated on MS medium, supplemented with 0.27 % phytagel, 1h before infiltration. Agrobacterium cultures transformed with the binary gateway constructs were grown overnight in 100 ml YEB supplemented with 10 mM MES and 20 µM acetosyringone at pH 5.6, and rifampicin (100 mg/L) and spectinomycin (100 mg/L) antibiotics. Bacterial cells were collected and resuspended in 20ml MMA medium (MS supplemented with vitamins, 10mM MES, 2% sucrose, 200 µM acetosyringone, pH 5.6) and incubated on a shaker at 22° C for 1h. Infiltration occurred by dipping the onion tissue in the bacterial suspension and subsequent vacuum infiltration for 20 minutes with shaking. After releasing the vacuum, plant material was washed in sterile water and incubated on MMA medium for cocultivation at 22° C in a 16h/8h photoperiod for 3-4 days. One day before GFP-expression analysis, the infiltrated plant tissue was incubated in 20mM PIPES-KOH buffer pH 7 for 24h with shaking.

BY-2 cell line transformation

At day 1 of the transformation protocol, a wild type BY-2 culture in stationary phase was diluted 10 times in 40 ml BY-2 medium (Nagata and Kumagai, 1999). On the same day a 10 ml YEB culture with the appropriate antibiotics of the Agrobacterium tumefaciens
LBA4404(pVirG) strain, carrying the Gateway plasmid, was started to grown at 28° C. Two days later the A. tumefaciens culture was diluted 10 times in 10 ml YEB without antibiotics and further incubated at 28° C. On day 4, 4ml of the mid-log phase BY-2 culture was decanted gently into an 8 cm sterile Petri dish after which 100 µl of the A. tumefaciens culture was added and gently mixed. This mixture was incubated in the dark at 25-28° C without shaking and after two days of co-cultivation, the mixture was spread on a plate with solid BY-2 medium containing 100 mg/L kanamycin (to select for transformed calli), 500 mg/L Cb and 200 mg/L vancomycin to kill the Agrobacteria. After approximately two weeks, single emerging calli were transferred to fresh BY-2 plates containing the same antibiotics. These clonal calli could be distinguished from chimeric calli based on their homogeneous growth pattern and were subcultured on plate several times. To check the potentially transformed calli, 20 ml liquid cultures were started from calli of 1 cm diameter. Next, log-phase cultures were assayed for expression of the transgenic constructs via immunoblot analysis. Selected transformed BY-2 lines were kept on solid BY-2 plates without antibiotics.

Further propagation of the BY-2 cell lines in liquid cultures and on plate was performed according to the protocols described in Nagata and Kumagai (1999). Subculturing of liquid cultures was performed by making a 1:100 dilution of a culture in stationary phase in fresh medium every week.

Protein extraction (using extraction buffer with the same composition as the buffer used for preparation of microsomal fractions without sucrose) and concentration measurement was performed as described above, 30-50 µg of total protein was concentrated using 500 µl centrifugal concentrators according to the instructions of the manufacturer (Millipore Corporation, Bedford, MA) and loaded for SDS-PAGE and western analysis.

**Chitin binding assay**

For the activation and regeneration of chitin, practical grade chitin from crab shells (Sigma, St. Louis, MO) was added to an equal volume of distilled water. The chitin was ionically activated by first adding 4 M NaOH solution (2 ml per 200 ml chitin/ water suspension) while gently stirring. After 30 minutes, the hydrated powder was sieved through four layers of miracloth (Biosciences) and extensively washed with tap water. Second, an equal volume of distilled water and 4 M HCl (2 ml per 200 ml chitin/ water suspension) was added to the powder and after 30 minutes the powder was again sieved and washed with tap water.
Next, the powder was resuspended in distilled water and this suspension of regenerated chitin was used to make chitin columns.

Freshly grown transformed BY-2 cultures were grinded in liquid nitrogen and resuspended in extraction buffer consisting of 50 mM sodium acetate and 0.2 M NaCl at pH 5. After filtration and centrifugation, the protein content of the supernatant was determined (see above). The subsequent steps were all performed at 4° C. 50 µg of total protein in 200 µl was loaded on the chitin column and incubated for 30 minutes. Unbound proteins were washed off twice with 2 ml extraction buffer. Finally, the bound proteins were eluted with 1.5 ml 100 mM acetic acid (pH 2.8) and collected in a tube containing 50 µl 1 M Tris buffer. Fractions were concentrated and half of the amount of the samples was analyzed by immunoblot analysis. As a control, an equal amount of protein was loaded on an empty column, collected and assayed in the same way as the other fractions. As a positive control for chitin binding, the stinging nettle lectin UDA was used (Pneumans et al., 1984), which binds in a pH-interval of 4.0-8.0. Chitin binding was performed with 1 µg of purified UDA lectin in 50 mM sodium acetate buffer (pH 5) 0.2 M NaCl and 1 ml of 100 mM acetic acid, pH 2.8. Fractions were collected, concentrated and analyzed by SDS-PAGE followed by coomassie staining.

**NF degradation / protection assay**

Protein extracts of wild type and Srchi24-overexpressing BY-2 cultures were prepared as described above. 14C-labeled Nod Factor fractions PI (containing a C18:1 or C16:0 fatty acid) and PII (containing C18:0 fatty acid) of A. caulinodans were prepared and purified as described by Mergaert et al. (1993). Radioactive incorporation was measured by scintillation counting and both NF fractions were used and gave similar results. A set of different incubation conditions was tested: 0.1, 10 or 100 µg of total protein extract were incubated with the 14C-labeled NFs (3000 counts per minute (cpm)) in 200 µl buffer for 30 minutes, 2, 4, 8, or 18 hours at 0° C or 37° C. After n-butanol extraction, NFs and their degradation products (non-reducing end) were analyzed by reversed thin-layer-chromatography (TLC) as described by Mergaert et al. (1993). Visualization and analysis of the reaction products was done with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**MBP-Srchi24 protein synthesis and purification in E. coli**

For expression of MBP-Srchi24 and MBP proteins, E. coli (MC1061) cells containing pMalc2-Srchi24 or pMalc2, respectively, were grown in Luria-Bertani medium (1 % trypton, 1 %
NaCl, 0.5 % yeast extract, and 0.1 % glucose) at 37° C until cell density measured at 600nm reached a value of 0.4 to 0.6. Next, isopropyl β-D-thiogalactosidase (IPTG) was added to a final concentration of 0.1 mM, and the cells were grown for another 2h at 30° C. Subsequently, cell lysis of the bacteria was performed by using ultrasonic waves, and the cell debris and supernatant were separated by centrifugation. The soluble protein fractions were then enriched for the MBP or MBP-Srchi24 by affinity chromatography according to the manufacturer’s instructions (New England Biolabs). Proteins were eluted in phosphate buffered saline (PBS) medium (pH 7.4).

**Pichia transformation**

Linearized pPICZalfa(Scfv-Fc) and pPICZC(Srchi24) vectors were transformed in Pichia pastoris GS115 by electroporation as described in the manufacturers instructions (Invitrogen). Transformed yeast colonies were selected on zeomycin and tested by PCR screening. Culture growth and induction of recombinant protein were done according to the Easyselect P. pastoris instruction manual (Invitrogen).

**Phage Display Library screening and antibody affinity analysis**

Screening of the library, ELISA assays and ScFv periplasmic fractionation and immunoblotting analysis was done according to Eeckhout et al. (2004).

**Antibody production and protein detection in P. pastoris**

For expression of the antibody, 4 different clones of the transformed Pichia pastoris liquid cultures were grown for 48h and 72h. After centrifugation of the cells, the supernatant was resuspended in 2x TGS sample buffer with or without 100mM β-mercapto-ethanol, boiled for 5 min at 95° C and loaded on protein gel. Silver staining of the gel was performed for detection of the proteins in the growth medium (see above), or immunoblot analysis was performed (as described above) by using human anti-Fc antibody (diluted 1/2500).

**Protein A purification of ScFv-Fc antibody and immunoprecipitation**

Yeast growth medium containing the ScFv-Fc antibody was brought to pH 8.0 through addition of 10 ml Tris buffer pH8 per 100 ml supernatant. Subsequently, precipitation was performed by adding 20 g (NH₄)₂SO₄ to 50 ml supernatant, and incubation at 4° C for 2h while stirring. The mixture was centrifuged for 15 minutes at 1200xg at 4° C and the pellet
was resuspended in 2 ml 25 mM Tris pH 8.0, which was supplemented with proteinase inhibitors. The protein concentration was then estimated by dot-spot analysis.

Dot-spot analysis was performed by spotting 7 µl of a range of 8 dilutions on a Hybond-C-nitrocellulose membrane strip of 0.5 cm – 9 cm. When dry, the strip was blocked with 3 ml 2 % skim milk in PBS-Tween (0.05 %) for 1h at room temperature. Next, the strips were washed 3 times with PBS-T and incubated for 1.5h at room temperature with the primary human anti-Fc IgG antibody coupled to HRP 2% skim milk in PBS-T. After subsequent washing, HRP was detected using the chemiluminescence detection kit according to the manufacturers instructions (Perkin-Elmer Life Sciences, Inc., Boston, MA). As a standard, the IgG1 purified antibody was analyzed in a range of known protein amounts.

For purification of the antibody, protein A beads (binding capacity of 20 mg antibody per mg powder) were prepared through mixing an equal volume of protein A powder with distilled water on a glass filter. Subsequent washing was done with distilled water (20 ml per 0.1 g). The protein A beads were then added to the binding buffer (25 mM Tris) in a volume ratio of 25 % buffer / 75 % beads. The enriched protein extracts obtained by ammoniumsulphate precipitation were mixed with a sufficient amount of protein A beads and incubated overnight while stirring at 4° C. Before and after binding, the bead supernatant was tested for the presence of unbound antibody by dot-spot analysis (see above). After 2 minutes of centrifugation at 2000xg, the supernatant was discarded, and the beads were washed in 25 mM Tris buffer for 5 times. Elution of the antibody was performed by adding 0.1 M glycin pH 3.0, centrifuged shortly, after which the supernatant was supplemented with Tris pH 8.0 buffer (100 µl/ ml elution) and the concentration of pure antibody was estimated by dot spot analysis.

Immunoprecipitation was performed to test the specificity of the ScFv-Fc monoclonal antibody for the Srchi24 protein by using protein extracts of Srchi24-overproducing BY-2 cells, wild-type BY-2 cells, and wild-type BY-2 cells supplemented with 100 ng of pure MBP-Srchi24 protein. A mix of 50 µl protein A beads and 200 µl (5 µg) of pure ScFv antibody (see purification step) was pre-incubated for 2h at room temperature for binding of the antibody to the protein A beads. This mixture was incubated with 0.5 ml (500 µg) protein extract for 2h at room temperature. As a control for unspecific binding to the protein A beads, extracts were also incubated with protein A that was not pre-incubated with antibody. After centrifugation, the beads were washed in 25 mM Tris buffer for 4 times and the pellet was resuspended in 25 µl 1x TGS sample buffer (Novex), supplemented with 100mM β-
mercapto-ethanol, boiled for 5 min at 95° C and loaded on protein gel. Immunoblot analysis was performed using the polyclonal antibody against MBP-Srchi24 (see above).

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CHAPTER 4

Transformation of *Sesbania rostrata*
COVER: Stereomicroscopical image of in vitro regenerated S. rostrata shoot after transformation and cultivation of hypocotyl tissue with the Agrobacterium tumefaciens pFT10-GUS strain. Blue staining is observed in the left transgenic shoot.
INTRODUCTION

The S. rostrata - A. caulinodans symbiosis remains an intriguing model for studying the nodulation process (see Chapter 2). The molecular attempts to unravel nodulin gene functions, such as changing gene expression levels by the application of gene silencing or ectopic expression, identifying the sites of promoter-activity by the use of promoter-reporter constructs, etc, is only feasible by the use of transgenic plants. The model legumes M. truncatula and L. japonicus are known to be transformable (Trieu and Harrison 1996, Trinh et al. 1998, Aoki et al. 2002), but S. rostrata belongs to the recalcitrant legume species (Vlachova et al., 1987). S. rostrata transformation is especially interesting because this water tolerant legume can be nodulated on both the stem and on the root, and is capable of using two different tracks for rhizobial invasion, the LRB and the RHC invasion depending on the growth conditions, and resulting in determinate and indeterminate nodules, respectively.

Alternatively, S. rostrata transgenic roots can efficiently be obtained using Agrobacterium rhizogenes. The A. rhizogenes 2569 strain induces the formation of ‘hairy roots’ upon infection of hypocotyls of embryonic axes of S. rostrata. The A. rhizogenes strain used for infection carries the bacterial rol gene-containing Ri plasmid, but is also transformed with a binary vector enclosing a selection marker and the gene of interest between the T-DNA borders. The naturally mediated T-DNA transfer to the infected plant cells results in the formation of co-transformed roots with an efficiency of 70% (Van de Velde et al., 2003). In vivo screening for co-transformed roots is performed by the use of an egfp marker gene, and only requires oxygen as a substrate to emit green fluorescence under UV light (Haseloff et al., 1999). The transgenic roots nodulate normally, although a bigger amount of nodules at the lateral root bases is observed in comparison to wild type nodulation in hydroponic conditions, probably because of the heavy branching of the transformed roots. Furthermore, the CaMV 35S promoter is constitutively active during nodule formation, making it a suitable promoter for ectopic gene expression during nodulation (Van de Velde et al., 2003). In conclusion, this is a very useful, efficient and fast transformation method to unravel gene functions that are proposed to be involved in LRB and/ or RHC root nodulation.

Unfortunately, the most attracting feature of S. rostrata, namely the synchronous nodule development at the predetermined adventitious root primordia on the stem cannot be examined in these transgenic plants. Therefore, a major breakthrough for the study of nodulation in this water-tolerant legume would be to find a method for obtaining transgenic
S. rostrata plants. Previous research challenged already several transformation methods, but regeneration of Agrobacterium tumefaciens-transformed S. rostrata tissue was very difficult or impossible (Vlachova et al., 1987; Pellegrineschi and Tepfer, 1992).

The regeneration problem was attempted to be circumvented by the application of optimal hormonal concentrations to the growth medium. Exogenous application of 0.2-0.5 mg/L benzylaminopurine (BAP) induces callus formation, and callus formation was more efficient with 5mg/L alpha-naphthalene acetic acid (NAA) or 2mg/L dichlorophenoxyacetic acid (2,4-D), although these calli never regenerated to shoots (Vlachova et al., 1987; S. Goormachtig, unpublished results). Hypocotyl tissue could initiate shoot formation from the inner cell layers after one month of incubation on 1mg/L BAP and 0.05-1mg/L indole-butyric acid (IBA) (S. Goormachtig, unpublished results). Pellegrineschi and Tepfer (1993) achieved shoot formation out of S. rostrata calli through modification of the photoperiod, nutritive compounds and phytohormones. For root formation, no additional hormones are needed.

In this study we attempted to obtain S. rostrata transgenic plants by applying a recently developed transformation system, the multi-auto-transformation (MAT) vector system (Ebinuma et al., 1997b). This system, based on Agrobacterium transformation, uses the morphological changes caused by the oncogenes of Agrobacterium as selection marker. By putting the oncogenes between removable elements which are excised by homologous recombination after transformation, the transgenic plants can be made marker-free (with normal phenotype) without the need of sexual crossings.

Two systems were developed for using the MAT vector system. They differ from each other by the use of different oncogenes. The first system makes use of the rol A, B & C genes of A. rhizogenes 1724 as selection marker. The hairy root phenotype can be applied as marker for transformed plant cells and after excision of the rol genes, spontaneous regeneration occurs out of the green segments of the hairy roots (Ebinuma et al., 1997a; Cui et al., 2000; Cui et al., 2001). The second system works with the A. tumefaciens isopentenyltransferase (ipt) gene as a selection marker causing an extreme shooty phenotype (ESP). Shoots with abnormal phenotype have wrinkled leaves, loss of apical dominance and shortened internodes. This phenotype is easy to detect, but the oncogenes have to be removed because rooting is impossible due to the ipt expression. To obtain normal shoots, the ipt-expression cassette is excised by site-specific recombination (Ebinuma et al., 1997a & b; Sugita et al., 2000).

After transformation, regeneration and excision, three kinds of transgenic plants are observed: marker positive plants, marker-free plants and somatic mosaics or chimeric
plants. The longer the cultivation time, the more transgenic cells show excision of the ipt-cassette, so more marker-free shoots regenerate. As the ipt gene function is dominant, chimeric plants would likely show ESP. The rol system also produces a lot of chimeric plants, and it is difficult to select non-chimerical marker-free transgenic plants visually using the rol genes in the R₀ generation. By crossing chimeric plants, segregation of non-chimeric progenies takes place with a high frequency. Hence, the MAT vector system can generate non-chimeric marker-free transgenic plants without sexual crossings, although they are produced more efficiently through crossings (Ebinuma et al., 1997a).

The system has the benefits that the oncogenes are not only used as a selection marker, but also as an endogenous supply of auxins or cytokinins, increasing the regeneration potential of recalcitrant plant species through internal manipulation of cytokinin-to-auxin ratio. Another advantage is the occurrence of marker excision whereby negative selection is circumvented. In other transformation methods the selective agents decrease the ability of plant cells to proliferate and differentiate, create the uncertainty of environmental impacts on many selectable marker genes, and are difficult to carry out multiple transformations to increase the number of the desired genes by using the same selectable marker (Ebinuma et al., 1997a & b).

Because S. rostrata easily develops transgenic roots after infection with A. rhizogenes, the first system has a high potential to succeed. However, regeneration from hairy roots has not been achieved so far, a difficulty that might be resolved by the excision system. Also the system with the ipt-gene induction might work because S. rostrata displays high endogenous auxin levels which could be causal to its recalcitrance. S. rostrata produces adventive roots on the stem and easily develops roots. Thus by increasing the endogenous cytokinin concentration, the auxin/cytokinin balance might be more prone to transformation.

The introduction of a control vector with the rol or ipt genes (Figure 1A) allows to determine the amount of constitutive expression of the rol or ipt genes, and to explore the hairy root or shooty phenotype, respectively. The use of these phenotypes as selection markers requires optimal conditions, which are examined by using different cured Agrobacterium strains, different plant tissues and different co-cultivation conditions. In optimal conditions, excision can be tested by using the excision vectors, pEXM120 and pEXM2 (Figure 1B), which contain a ‘hit & run’ cassette between a 35S promoter and a gus gene. Excision events can be observed by blue staining, PCR or Southern analyses. After optimization of the system, the MAT vector (Figure 1C), with the gene of interest, can finally be introduced and marker-free transgenic plants can be obtained.
RESULTS AND DISCUSSION

Optimization of the transformation conditions

Several plant tissue types were tested, hypocotyls and cotyledons of one week old seedlings, and embryonic axes prepared as described in Van de Velde et al. (2003). Transformation was performed by using various Agrobacterium tumefaciens strains (Hellens and Mullineaux, 2000), A. tumefaciens LBA4404, C58C1(pMP90), and C58C1(PGV2260). Infected plant tissue was co-cultivated for 2 or 5 days, either with 24h dark or with 16h/8h light/dark photoperiods at 22°C. Optimal conditions were screened for both systems through transformation with Agrobacterium strains containing the pROL20 or IPT10 control vector (Figure 1A) and evaluated based on the transformation frequency obtained in the different conditions through GUS-staining and on the observation of oncogenic phenotypes.

The rol system

The results of gus-staining on the transformed explants with Agrobacterium tumefaciens strains containing the pROL20 control vector are summarized in Table 1. For the application of A. tumefaciens LBA4404 and C58C1(pMP90), 44 explants were tested for each type of plant tissue in all conditions described and no transformed explants were found (data not shown). A. tumefaciens C58C1(pGV2260)(pROL20) transformation resulted in GUS-positive explants with different transformation frequencies depending on the conditions (Table 1). To screen for the most optimal conditions, 84 embryonic axes, 72 cotyledons, and 72
hypocotyls were transformed and co-cultivated in various photoperiods (16/8h light or 24h dark) during 2 or 5 days. Transient expression was analyzed by GUS-staining of the explants and the highest efficiency (17.8%) was obtained when embryonic axes were co-cultivated for 2 days in a light regime of 16h light/ 8h dark at 22°C. In these optimal conditions, another 496 explants were transformed with A. tumefaciens C58C1(pGV2260)(pROL20) to obtain explants that should develop hairy roots upon expression of the rol genes. However, further incubation of the infected explants for 4 to 7 weeks never led to hairy root development. Additional efforts to enhance transformation efficiency were performed by introducing a ternary pVirG helper plasmid in A. tumefaciens C58C1(pGV2260)(pROL20) (Van der Fits et al., 2000), and by exogenous application of the ethylene inhibitor compound aminoethoxyvinlyglycine (AVG) (7µM) during the co-cultivation (Christey et al., 1997). Unfortunately, this increased neither the efficiency nor the potential for hairy root induction, so no further experiments were performed with the rol system.

<table>
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<tr>
<th>A. tumefaciens strain</th>
<th>Plant tissue</th>
<th>Cocultivation</th>
<th># Explants tested</th>
<th># GUS positive</th>
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<tr>
<td>C58C1(pGV2260)</td>
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<td>16/8L 2d</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16/8L 5d</td>
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<td></td>
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<td>5</td>
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<tr>
<td></td>
<td></td>
<td>24hD 5d</td>
<td>21</td>
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<td></td>
<td>total:</td>
<td>84</td>
<td>15 or 17.8%</td>
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<tr>
<td>C58C1(pGV2260)</td>
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<td>16/8L 2d</td>
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<td>4</td>
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<td></td>
<td></td>
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<td>8 or 10.8 %</td>
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<td>7 or 9.4%</td>
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TABLE 1: A.tumefaciens transformation of S. rostrata using the pROL20 control vector.
16/8L, 16 hour light and 8 hour dark cocultivation conditions; 24hD, 24 hour dark cocultivation conditions; 2d or 5d, 2 or 5 days of cocultivation, respectively.

These observations are in contrast with the highly efficient induction of hairy roots upon infection of S. rostrata embryonic axes with A. rhizogenes 2659 (Van de Velde et al., 2003). An explanation might be that the 10kb T-DNA transfer to the plant cells is interrupted. This would explain why we detect GUS-positive cells and no hairy roots. The transfer is initiated from the right border region where the gus gene is located and followed by the rol genes. Another explanation could be that the rol genes, which are derived from A. rhizogenes 1724, are not effective to affect S. rostrata auxin levels or to induce hairy root formation. The A. rhizogenes 1724 has a Ri mikiomine type plasmid (Moriguchi et al., 2001;
Shiomi et al., 1987), whereas the A. rhizogenes 2659 strain carries the cucumopine type Ri plasmid (Combard et al., 1987; Filetici et al., 1987; Davioud et al., 1988). Indeed, the A. rhizogenes 1724 strain caused no visible changes when used for infection on S. rostrata embryonic axes (Van de Velde et al., 2003).

**The ipt system**

The pIPT10 control vector was introduced in the different types of A. tumefaciens used for transformation of the S. rostrata explants. Table 2 demonstrates that by the use of the ipt system, transgenic calli and shoots were obtained (Figure 2), although with a low frequency. Pilot experiments with the pIPT10 control vector in the different conditions on the 3 different types of plant tissue were performed to define the optimal conditions. The rol system experiments displayed the best results for infection of the A. tumefaciens pGV2260 strain on embryonic axes, so fewer explants were analyzed with the A. tumefaciens LBA4404 and C58C1(pMP90) strains. In a first analysis, only embryonic axes were infected with the 3 Agrobacterium strains. Transformation with the A. tumefaciens C58C1(pGV2260)(pIPT10) strain resulted in a higher number of transformed cells compared to the other Agrobacterium strains. In Table 2 it is shown that the amount of GUS-positive explants after transformation with this strain is lower than with the other two strains, but the blue staining indicative for the amount of transformed cells was more pronounced and was comparable to GUS-staining of A. rhizogenes transformed tissue. This observations leads to the conclusion that also for the ipt system the A. tumefaciens C58C1(pGV2260) strain is the most appropriate for transformation of S. rostrata explants.

The different types of explants were subsequently analyzed after transformation with the A. tumefaciens C58C1(pGV2260)(pIPT10) strain, and in parallel the same strain containing an additional pVirG plasmid was used for transformation. Half of the transformed material was analyzed for transient GUS expression, and the other half was preserved in vitro for 7 weeks. The application of the ternary transformation method (with pVirG) increased the transformation frequency in all tissues tested, except for hypocotyl infection which showed the highest transformation efficiency (77%) without the use of pVirG. However, the hypocotyl infection with Agrobacteria containing the pVirG plasmid resulted in the development of transgenic shoots, which was not the case if the pVirG plasmid was not used. We could conclude that the most optimal conditions to obtain an acceptable transformation efficiency and subsequent shoot regeneration enclose infection of hypocotyl tissue with A. tumefaciens C58C1(pGC2260)(pVirG)(pIPT10) co-cultivated at 22°C for 2 days.
in a 16h/8h photoperiod on medium supplemented with 200µM acetosyringone. Moreover, three transgenic shoots were obtained after 7 weeks of in vitro propagation (Figure 2). The low frequency (3 shoots on 114 explants) might be the consequence of the fact that shoot organogenesis is initiated in the deeper cell layers of hypocotyls that cannot be reached by the bacteria (S. Goormachtig, unpublished data). An effort to transform hypocotyls that were longitudinally sectioned, which would allow bacteria to enter the deeper cell layers, did not succeed because the explants died.

When the transformed hypocotyls were supplemented with 2mg/L NAA and 0.1mg/L BAP one month after transformation the frequency of calli formation and subsequent shoot regeneration increased. The optimal hormone concentrations were defined by looking into the literature on S. rostrata regeneration (see above) and by additional experimental data after testing several concentrations of BAP, IBA, IAA and NAA. In conclusion, the analysis of the IPT10 control vector demonstrated that in optimal conditions a high transformation frequency (60%) of S. rostrata plant cells is achieved. These transformed cells showed the capacity for calli and shoot formation, although with a lower frequency of 16 transformed calli and 3 transformed shoots on 114 explants, respectively.

<table>
<thead>
<tr>
<th>Plant tissue</th>
<th>Cocultivation</th>
<th># Explants tested</th>
<th># GUS positive explants</th>
<th>% GUS positive calli</th>
<th>% GUS positive shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>C58C1(pMP90) embr axes</td>
<td>16/8L 2d</td>
<td>15</td>
<td>6 or 40%</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td>LBA4404 embr axes</td>
<td>16/8L 2d</td>
<td>10</td>
<td>7 or 70%</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td>C58C1(pGV2260) embr axes</td>
<td>16/8L 2d; 5d</td>
<td>30</td>
<td>25, bigger blue surface</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>24h: 2d; 5d</td>
<td>15</td>
<td>0</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td>total:</td>
<td>45</td>
<td></td>
<td>25 or 55%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58C1(pGV2260)(pVirG) embr axes</td>
<td>16/8L 2d</td>
<td></td>
<td>more blue cells compared to the absence of pVirG</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>24h: 2d</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>total:</td>
<td>44</td>
<td></td>
<td>55%</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>C58C1(pGV2260) cotyledons</td>
<td>16/8L 2d; 5d</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24h: 2d; 5d</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>total:</td>
<td>26</td>
<td></td>
<td>11 or 42%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C58C1(pGV2260)(pVirG) cotyledons</td>
<td>16/8L 2d</td>
<td>24</td>
<td>&gt; 42%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>24h: 2d; 5d</td>
<td>0</td>
<td>not tested</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>total:</td>
<td>26</td>
<td></td>
<td>20 or 77%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C58C1(pGV2260)(pVirG) hypocotyls</td>
<td>16/8L 2d*</td>
<td>114</td>
<td>69%</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

TABLE 2: A. tumefaciens transformation of S. rostrata using the pIPT10 control vector.
16/8L, 16 hour light and 8 hour dark cocultivation conditions; 24hD, 24 hour dark cocultivation conditions; 2d or 5d, 2 or 5 days of cocultivation, respectively; *, 200µM acetosyringone was supplemented during co-cultivation.

**Excision of the selectable marker genes**

The ipt system excision vector pEXM2 (Figure 1B) was used in A. tumefaciens C58C1(pGV2260)(pVirG) to test the excision potential of S. rostrata transformed hypocotyl cells in optimal conditions (2d, 16h/8h light, growth medium supplemented with...
acetosyringone). However, on 35 hypocotyl pieces analyzed, no blue staining was observed after transient expression. It is possible that, although the expression of the recombinase is driven by a constitutive 35S promoter, the recombinase was not yet active and that the ‘hit and run’ cassette is not excised by the time of transient analysis.

In vivo selection of transgenic IPT shoots

The GUS reporter system has the disadvantage that plant material is destructed after substrate addition. This critically blocked a straightforward analysis of this system because the frequency for obtaining transgenic shoots was low, and further development of these few shoots was impossible after GUS-staining. Introduction of the egfp marker gene allowed in vivo screening. So, the gus reporter gene in the pIPT10 control vector was replaced by the egfp gene, also driven by a 35S promoter. Consequently, hypocotyls were infected in the optimal conditions with A. tumefaciens bacteria containing the pIPT10(35S-egfp) construct. A total of 200 explants were infected, which should result in at least 5 EGFP positive shoots considering the frequency of 3/114 transgenic shoots obtained with the pIPT10-GUS control vector. However, green fluorescent cells were less frequent observed compared to the GUS-assays. Nevertheless, the detection of EGFP-positive cells did occur in a small fraction of the infected hypocotyls (Figure 3A, B) but no EGFP-positive shoots were detected in any of the regenerating hypocotyls.
Regeneration of co-transformed hairy roots

Another try-out to obtain transgenic S. rostrata plants was performed with A. rhizogenes 2659, which vigorously induces transgenic roots on S. rostrata (Van de Velde et al., 2003). Twenty embryonic axes were infected with A. rhizogenes 2659 carrying the pPZP200 binary vector including an egfp reporter gene, which results in nicely developed EGFP hairy roots after 4 weeks of in vitro growth. Addition of several phytohormone compounds in different concentrations was tested to achieve regeneration of the hairy roots (data not shown). Finally, 8 weeks after infection, transgenic EGFP-positive calli (Figure 3C, D) were formed out of the hairy root tissue, but unfortunately no transgenic shoot regeneration was achieved, which is probably the consequence of the presence of the rol genes integrated in the DNA of the transformed cells.

CONCLUSIONS

The rol system did not induce transgenic hairy roots on S. rostrata, but in the best conditions a number of GUS-positive cells were observed. The ipt system could be optimized in such a way that 3 transgenic shoots were obtained out of 114 infected hypocotyls. This is the first time that transgenic S. rostrata shoots were obtained. The introduction of the ipt gene in S. rostrata results in a higher transformation efficiency, which is probably acquired by changing the hormone balance in this aquatic legume plant, which entails high endogenous auxin concentrations. The introduction of the ipt gene, encoding the first enzyme of the cytokinin biosynthesis cascade, could perturb the high auxin/cytokinin balance in S. rostrata to a level that allows shoot development. Application of exogenous cytokinin could not cause this effect on S. rostrata (S. Goormachtig, unpublished results).

Thus, S. rostrata is transformable, though, only in tightly regulated conditions and with a low frequency. The application of the ipt cytokinin synthesis gene could aid in its transformability, but the MAT excision principle could not be controlled. Finally, the use of an
inducible promoter (Sugita et al., 2000; Ebinuma and Komamine, 2001) to tightly regulate the recombination might improve the system, although the recombination-efficiency could again reduce the shoot-formation efficiency. Therefore, it is probably better to switch to an alternative system in which the expression of the ipt gene is controlled by an inducible promoter. Switching on and off the promoter activity is in that case sufficient to initiate and attenuate the extreme shooty phenotype and achieve normal regenerated S. rostrata transgenic plants for which stem nodulation can be investigated. Hence, a good inducible promoter candidate system should be available for S. rostrata (see Chapter 3).

The problem of regeneration was solved by the application of optimal hormonal concentrations to the growth medium, and transformation efficiency was elevated by introducing an ipt gene in S. rostrata hypocotyls. It was shown that S. rostrata hypocotyl tissue can regenerate to shoots directly or via callus formation. However, direct shoot formation probably initiates in deeper cellayers that are not transformable, whereas the calli formed on the peripheral side of the hypocotyl pieces showed a higher transformation capacity. Hence, increasing callus formation and subsequent regeneration should be improved for an efficient production of transgenic shoots. So far, the transformation capacity of S. rostrata, using Agrobacterium tumefaciens-mediated transformation, is extremely low and certainly not ready for application in molecular research.

**METHODS**

**S. rostrata Brem. plant material: seed sterilization and germination**

Sesbania rostrata Brem seeds were sterilized as described by Goethals et al. (1989) and germinated on Petri dishes containing 0.8% (w/v) agar in tap water in the dark at 28°C for 2 days.

**Bacterial strains and constructs used for S. rostrata transformation**

A. tumefaciens strains LBA4404, C58C1(pMP90), and C58C1(PGV2260) (Hellens and Mullineaux, 2000) were grown at 28°C in yeast extract broth (YEB) medium (Sambrook et al., 1989) in the presence of the appropriate antibiotics: rifampicine (100 mg/L) for selection of the A. tumefaciens strain, gentamycin (25 mg/L) for selection of the pMP90 and pVirG plasmids, and kanamycin (50 mg/L) for selecting for the pROL20, pPT10 or pEXM2. The vectors pPT10, pROL20, pEXM2, pEXM120 and pMAT101 were provided by the MAT Vector Association (http://www.np-g.com/about/research/topix_study/mat/matvector_03.html) and
introduced in the A. tumefaciens strains by electroporation. The IPT10 T-DNA region contains a gus gene behind the CaMV 35S promoter, and this 35S-gus fragment was replaced by the 35S-egfp cassette through enzymatic digestion of the IPT10 vector with SacI and subsequent insertion of the HindIII blunted p35S-egfp fragment (Clontech, Palo Alto, CA, USA). The resulting plPT10(35S-egfp-T) plasmid was introduced in A. tumefaciens by electroporation.

A. tumefaciens-mediated transformation
A preculture was grown overnight and transferred to 100 ml liquid medium for further growth until an optical density of 0.6 was reached. The bacterial culture was supplemented with 100 μM acetylsyringone (Sigma-Aldrich, St. Louis, MO, USA) 4-5h prior to transformation. Bacterial cells were collected by centrifugation and resuspended in 20 ml YEB. Hypocotyls and cotyledons from plants germinated for 2 days in the dark followed by 5 days of growth at 21° C in 16h/8h light/dark regime, were cut just prior to Agrobacterium infection. Embryonic axes were prepared as described by Van de Velde et al. (2003). The plant tissue was wounded with a sterile needle at the height of the hypocotyls of the embryonic axes, at the ‘curvature’ of the hypocotyls, or at the surface of the cotyledons and subsequently incubated for 5 minutes in the concentrated bacterial suspension. Explants were cocultivated at the indicated conditions (see above and Table 1 and 2) on Petri dishes containing Murashige and Skoog (MS) medium supplemented with vitamins (Petit et al., 1987), 3 % (w/v) sucrose, and 0.27 % (w/v) Phytagel, and when applicable 200 μM acetylsyringone and/or 7 μM AVG. After 2 or 5 days of co-cultivation, the explants were transferred to new MS containing Petri dishes supplemented with 100 mg/L cefotaxime (Cf) and 500 mg/L carbenicillin (Cb) to kill the proliferating Agrobacteria. Every 2 weeks, the explants were transferred to new medium and after 1 month, they were transferred to medium supplemented with 2.5 mg/L IAA or 2 mg/L NAA and 0.1 mg/L BAP to increase the formation of callus and the regeneration frequency.

A. rhizogenes transformation of S. rostrata
This transformation method was performed as described in Van de Velde et al. (2003).
Histochemical localization of β-glucuronidase activity

GUS assays on the infected explants were done as described previously (Van de Eede et al., 1992). The presence of blue cells was analyzed with a stereo microscope MZFLIII (Leica, Wetzlar, Germany).

Visualization of EGFP fluorescence

In vivo screening of transformed cells for EGFP expression was done as described by Van de Velde et al. (2003).
CHAPTER 5

Targeted protein degradation via SINA proteins controls lateral root number and is essential for nodule formation in *Medicago truncatula*

Adapted from “Targeted protein degradation via SINA proteins controls lateral root number and is essential for nodule formation in *Medicago truncatula*”
Transmission electron microscopy picture showing rhizobia residing in the infected plant cells of a 35S:AtSINAT5DN M. truncatula young nodule. Symbiosome formation is hampered which is indicated by the large symbiosome space and the initial signs of bacterial degradation.
INTRODUCTION

Plant development and responses to the environment are often regulated by ubiquitin-mediated proteolysis of regulatory proteins (Ellis et al., 2002; Hare et al., 2003; Devoto et al., 2003; Zeng et al., 2006). These proteins, frequently repressors, are ubiquitinated and degraded via the 26S proteasome. The ubiquitination is performed by a sequential action of 3 enzymes: the ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes (Glickman and Ciechanover, 2002). The E3 ligases confer the specificity of degradation by selecting the target protein for ubiquitination. Several types have been identified in plants, such as the anaphase-promoting complex (APC), the Skp-Cullin-F-box (SCF) complex, the HECT-domain E3s, the RING E3s, and the U-box proteins (Callis and Vierstra, 2000; Joazeiro and Weissman, 2000; Estelle, 2001; Stone et al., 2005). The presence of a RING finger motif is common among all types of E3 ligases, with exception of the HECT-domain proteins. The RING domain is thought to mediate the interaction with E2 enzymes to facilitate the transfer of ubiquitin to a lysine of the target protein (Freemont, 1993). Within the SCF complexes, the F-box containing proteins select the protein for degradation. A total of about 700 F-box proteins have been identified in Arabidopsis thaliana (At) showing that those complexes play important regulatory roles (Gagne et al., 2002).

Auxin-mediated signaling for lateral root induction in A. thaliana implements the action of several E3 ligases. The SCF^{TIR1} E3 ligase complex, containing the TIR1 F-box protein, functions as auxin receptor (Dharmasiri et al., 2005), and targets the AUX/IAA repressors for degradation thereby activating gene expression (Gray et al., 2001). Mutants which perturbed SCF^{TIR1} complex formation, such as the tir1 and solitary root (slr) mutants, show several auxin signaling defects such as defects in lateral root formation (Ruegger et al., 1998; Fukaki et al., 2002, 2005; Vanneste et al., 2005). Another E3 ligase, XBAT32, is also induced by auxin and positively influences lateral root formation (Nodzon et al., 2004). The SINAT5 E3 ligase attenuates the auxin-induced lateral root formation as ectopic expression of the gene results in a lower number of lateral roots (Xie et al., 2002). The gene is located on chromosome 5 of the A. thaliana genome and encodes a protein of 309 amino acids. The N-terminal RING finger domain is followed by the conserved SINA domain. SIAH, which is the mammalian ortholog of SINAT, shows E3 activity after dimer formation although also a SIAH-containing SCF-like complex was characterized to be functional in ubiquitination (Santelli et al., 2005). SINAT5 acts upon dimerization, which is RING-independent and is essential for ubiquitin E3 ligase activity (Xie et al., 2002). Furthermore,
ectopic expression of a dominant-negative Cys49→Ser RING domain mutant of SINAT5 (SINAT5DN) causes more lateral roots compared to wild type plants. AtSINAT5 is involved in lateral root formation by specifically ubiquitinating the NAC1 transcription factor, which is a member of the NAM/CUC family of transcription factors and controls lateral root dependent gene expression (Xie et al., 2000, 2002).

Besides lateral roots, legume plants develop another type of secondary root organ, the nodule. Nodules arise after a compatible interaction with soil bacteria collectively referred to as rhizobia (Crespi and Galvèz, 2000). Medicago truncatula forms nodules after a complex signal exchange with its microsymbiont Sinorhizobium meliloti (Gage, 2004). Recognition of bacterially produced Nod factors switches on the nodule formation pathway which consists of two processes, rhizobial infection and organ development. The rhizobia enter the plant via curling of root hairs. Within the curl, cell wall hydrolysis and invagination of the cell membrane result in the formation of an infection thread (IT) which guides the bacteria towards deeper cortical cells. Meanwhile a nodule primordium (NP) is formed via re-initiation of cell division in the cortex and the pericycle (Timmers et al., 1999). The ITs reach the NP and bacteria are taken up by the cells of the primordium, differentiate into bacteroids that are surrounded by a plant-derived peribacteroid membrane and start to fix atmospheric nitrogen. At the same time, an apical meristem is installed and an indeterminate nodule develops consisting of different developmental zones: the meristem, the infection zone where bacteria are taken up by meristem-derived nodule cells, the fixation zone where N₂ fixation takes place and the senescence zone where both partners degrade (Vasse et al., 1990).

When plants interact with micro-organisms, not only during symbiotic interactions such as nodulation or mycorrhization, but also during pathogenic interactions, they try to control the extent of invasion and regulate the initiation of developmental changes (Mathesius, 2003). Therefore, plant responses to micro-organisms might involve pathways which have been ‘hijacked’ from plant developmental pathways as proposed by Nutman in 1948 and as has been intensively discussed in literature (Mathesius et al., 1998; 2000; 2003; deBilly et al., 2001; De Carvalho-Niebel, 2002; Ferraioli et al., 2004; Kondorosi et al., 2005). Nodule and lateral root formation show common aspects during development. Both organ formations display the same radial position, opposite the protoxylem poles. However, along the longitudinal axis of the root, nodules arise in zone I, i.e. just above the root tip where root hairs susceptible for infection develop, whereas lateral root primordia are initiated in the cells passing the elongation and young differentiation stages (Dubrovsky et
al., 2000). The development of both organs implies the initiation of a new vasculature, which is located centrally in a lateral root and peripherally in a nodule. For lateral roots, cell division is initiated in the pericycle, whereas for nodule formation divisions are initiated in both the pericycle and the cortex (Timmers et al., 1999). Also the number of organs is partially controlled by a similar mechanism as legume mutants with a defect in the autoregulation mechanism that controls the nodule number also show a change in lateral root number (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003). Additionally, NFs stimulate lateral root formation using the same signaling cascade as for the initiation of nodule formation (Olàh et al., 2005).

Here we describe the effect of ectopic expression of AtSINAT5 and AtSINAT5DN on lateral root and nodule formation in M. truncatula. Similar effects on lateral root formation were observed as have been described in A. thaliana (Xie et al., 2002), indicating that the AtSINAT5 protein is active in M. truncatula. Furthermore, in plants ectopically expressing AtSINAT5DN, a fix phenotype was observed due to an effect on symbiosome formation. Yeast-two-hybrid analysis revealed several interacting proteins of AtSINAT5 in M. truncatula roots and nodules, some of which were SINA-domain containing proteins. Moreover, nodulin proteins such as the leghemoglobins were shown to interact with AtSINAT5 in the yeast-two-hybrid screen. Our results suggest that AtSINAT5-like E3 ligase activities in M. truncatula regulate the number of lateral roots and additionally are involved in three steps of the nodulation process, at the stage of infection thread growth, during symbiosome differentiation, and in senescence, by controlling various nodulation-related proteins.

RESULTS

In silico identification of M. truncatula SINA proteins

TBLastX analyses using the M. truncatula MTGI EST database of The Institute for Genomic Research (TIGR) (http://tigrblast.tigr.org/tgi/) revealed six SINA domain-containing Tentative Consensus (TC) and 3 singleton EST sequences (Table 1). The derived amino acid sequences of MtSINA1 to -6 and of the AL377008 singleton showed 70% or more similarity to AtSINAT5, while the amino acid sequence derived from 2 other singletons displayed less than 60% similarity. All the encoded protein sequences displayed the conserved RING finger and SINA domain (Figure 1). The MtSINA6 sequence contained a shorter SINA domain, which was confirmed in the genomic sequence (data not shown). Also the MtSINA3 C-terminal part is shorter and less conserved.
FIGURE 1: Aligned protein sequences of *M. truncatula* SINA-1 to SINA-6 and AtSINAT5. All 7 proteins contain the RING finger motif (labeled by *), and have a SINA domain (labeled by $). Black and grey shaded amino acids are identical and similar, respectively, for 70% of the sequences.

The MTGI TIGR databases also allow to perform an initial in silico expression analysis (Journet et al., 2002; Lamblin et al., 2003). The representation of a clone within a specific library is presented in Table 1 as the percentage of that cDNA library. None of the clones corresponded to a gene which was specifically upregulated during nodulation. A cDNA clone
of MtSINA3 and of MtSINA6 has been found in nodulation related libraries among others and two from the three singleton ESTs were isolated from nodulation related libraries: BE997651 from a senescent nodule library (GVSN) and AL377008 from a 4 days post-inoculation (dpi) nodule library (MtBB). The AL377008 singleton sequence is identical to part of the MtSINA5 TC sequence, but was not included in its tentative consensus of ESTs and therefore AL377008 is still considered as a singleton sequence (Table 1).

<table>
<thead>
<tr>
<th>Name/AC No.</th>
<th>In silico Northern expression</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtSINA1 TC102374 AC150444.7</td>
<td>Mycorrhizal roots (MHAM) (0.03), Developing flower (0.03), Leaves infected with Colletotrichum trifolii (DStL)(0.02), Developing root (0.03), Methyl Jasmonate-Elicited Root Cell Suspension Culture (0.01)</td>
<td>89%</td>
</tr>
<tr>
<td>MtSINA2 TC102369</td>
<td>Drought (0.04), Elicited cell culture (0.02), Mycorrhizal roots (MHAM) (0.01), Developing leaf (0.01) and flower (0.02), Phosphate-starved leaves (0.01)</td>
<td>83%</td>
</tr>
<tr>
<td>MtSINA3 TC109024</td>
<td>Nematode infected roots (BNIR) (0.06), 3dpi S. meliloti inoculated roots (KV3) (0.02), Leaves infected with Colletotrichum trifolii (DStL)(0.02), Early seed development (GESD)(0.02)</td>
<td>76%</td>
</tr>
<tr>
<td>MtSINA4 TC102612 AC137828.24</td>
<td>Drought (0.03), Developing stem (0.03), N-starved root tips (MTBA) (0.01), Mycorrhizal roots (MBC and MHAM) (0.01)</td>
<td>90%</td>
</tr>
<tr>
<td>MtSINA5 TC104350</td>
<td>Mycorrhizal roots (MHAM) (0.01), Pathogen-induced, young trifoliate leaves (Phoma-infected) (0.03)</td>
<td>84%</td>
</tr>
<tr>
<td>AL377008 4dpi S. meliloti inoculated roots (MtBB)</td>
<td></td>
<td>84%</td>
</tr>
<tr>
<td>MtSINA6 TC109632 AC146590.25</td>
<td>3 dpi S. meliloti inoculated roots (KV3) (0.02), Mycorrhizal roots (MHAM) (0.06), Early seed development (GESD)(0.02), Pathogen-induced, young trifoliate leaves (Phoma-infected) (0.03), Aphid-infected shoots (0.02)</td>
<td>71%</td>
</tr>
<tr>
<td>CB893977 Oligogalacturan treated root</td>
<td>52%</td>
<td></td>
</tr>
<tr>
<td>BE997651 Senescent nodules (GVSN)</td>
<td>33%</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1: MtSINA sequences identified in TBLastX analysis with AtSINAT5 on M. truncatula EST databases. Columns show the name that was given in our study; the TC (or singleton) number and, if available, the AC number of the genomic clone; the in silico Northern provided in the TIGR MTGI database lists the cDNA libraries in which the ESTs are present and between "()" the percentage of expression in the total library; the percentage of amino acid similarity to AtSINAT5. In bold, all nodulation-related cDNA libraries.

Ectopic expression of AtSINAT5 and AtSINAT5DN in M. truncatula

Several ORFs were identified which were highly similar to AtSINAT5. However, in silico analysis did not define an MtSINA ORF that corresponded to a gene upregulated during nodulation. To unravel the role of SINA proteins in nodulation, we decided to ectopically express the AtSINAT5 and the dominant-negative form AtSINAT5DN in M. truncatula.

Leaves of two subspecies of M. truncatula, Jemalong J5 and R108, from which Jemalong was selected as model legume (Cook, 1999; Frugoli and Harris, 2001), have been used for Agrobacterium tumefaciens-mediated transformation of the 35S:AtSINAT5WT and 35S:AtSINAT5DN constructs (see Methods). The procedures are very similar for both plants but are much shorter (7 months compared to 1 year) for R108 to obtain F0 due to a shorter tissue culture period. The same phenotypes have been observed in the two subspecies and
because of this relative short generation period, most analyses shown here are performed using the R108 plants.

Nine different R108 lines transformed with 35S:AtSINAT5DN, containing 1 to 5 inserts as shown by southern hybridization (data not shown), were further analyzed. As shown in Figure 2A, leaves of the various lines contained different levels of AtSINAT5DN transcripts. For the J5 plants, 6 different lines were obtained, having different levels of AtSINAT5DN transcripts in the leaves (Figure 2B). For the 35S:AtSINAT5WT transformants, only R108 lines were obtained. Southern hybridization indicated 27 different T-DNA integration patterns resulting in various expression levels within the leaves (Figure 2B).

The different lines were grown for seed setting and segregation analysis of F1 plants was performed on glufosinate-ammonium (basta) selection (see Methods). Most lines showed a 3:1 segregation pattern, indicating that integration of the T-DNA occurred at a single locus. Southern blotting on 6 F1 J5 35S:AtSINAT5DN lines proved that the insert was stable in the F1 generation (data not shown).

Lateral root formation in M. truncatula 35S:AtSINAT5 and 35S:AtSINAT5DN plants
Ten basta selected plants from 13 different M. truncatula 35S:AtSINAT5 R108 lines were grown in vitro to analyze root and shoot growth (see Methods). As shown in Figure 3A, no obvious shoot phenotype was observed but the roots contained fewer lateral roots compared to wild type plants. The average number of lateral roots per cm of main root
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Figure 3: M. truncatula 35S:AtSINAT5WT lateral root phenotype. A, after 40 days of in vitro growth, the R108 35S:AtSina5WT plant (right) has a longer main root and fewer lateral roots compared to wild type R108 (left). B, the average number of lateral roots per cm main root (LRs/cm) on 10 plants of 6 35S:AtSINAT5WT lines (lane 4 to 9) compared to wild type (lane 1 to 3). Error bars: standard error measurements.

As shown in Figure 3B, ectopic expression of AtSINAT5 resulted in a significantly lower number of lateral roots per cm in all lines compared to wild type (see Methods). The average number of lateral roots was 1.33 ± 0.10 LRs/cm for 35S:AtSINAT5WT compared to an average of 2.75 ± 0.29 LRs/cm in the control plants (p < 0.001; see Methods).

35S:AtSINAT5DN plants were more vigorous compared to wild type. As shown in Figure 4A, after 8 weeks of growth in greenhouse condition in rich soils (see Methods), plants of several 35S:AtSINAT5DN lines were 1½ times the size of wild type plants. Besides having more shoots and roots, also the leaf size was larger compared to control leaves (Figure 4B). To analyze the effect on root growth in detail, plants showing a high level of transgene expression, as measured in leaf tissue (see Methods), were grown in vitro and the number of LRs/cm was determined (Figure 4D and E). Control plants had an average of 2.16 ± 0.51 LRs/cm whereas 35S:AtSINAT5DN transgenic plants had an average of 4.12 ± 0.57 LRs/cm (p<0.05). The effect was even more pronounced when plants were grown on a concentration of 1- alpha-naphthalene acetic acid to stimulate lateral root formation. As shown in Figure 4E, when 6 day old plants were transferred to 1-NAA containing medium for another 6 days (see Methods), an average of 4.95 ± 0.33 LRs/cm was observed whereas only 1.35 ± 0.29 LRs/cm were observed in control lines (p<0.001).

The effect of 35S:AtSINAT5WT and 35S:AtSINAT5DN on nodule number in M. truncatula

The analysis on lateral root growth showed that AtSINAT5 is active in M. truncatula. To analyze the effect on nodulation, plants of 27 different R108 lines ectopically expressing
AtSinat5WT were germinated on nitrogen-poor medium and inoculated with Rhizobium meliloti 41 (pPHC60-gfp) which can be visualized by fluorescence microscopy (Cheng and Walker, 1998; see Methods).

Nodule formation was analyzed at several time-points after bacterial inoculation using fluorescent stereomicroscopy. No differences compared to wild type plants could be observed and the transgenic lines finally had an average number of 6 nodules per plants which is a similar amount compared to wild type plants grown on plates (data not shown).

To analyze the effect of 35S:AtSINAT5DN on nodule formation, ten plants of seven transgenic lines were grown in perlite in nitrogen-poor conditions and inoculated with Rhizobium meliloti 41 (pPHC60-gfp). As shown in Figure 5, an average of 4.22 ± 0.29 nodules per plant was obtained for the 35S:AtSINAT5DN lines whereas control plants contained 8.53 ± 1.02 nodules per plant at 22 dpi (Figure 5A; p<0.001). Moreover, 40% of the nodules that appeared on 35S:AtSINAT5DN plants were white, which reflects the shortage of active leghemoglobin that normally colours the nodules pink (Figure 5B compared to 5F). The absence of leghemoglobin is indicative for a defective nitrogen fixation indicated as fix-. Analyzing those nodules by fluorescence microscopy to visualize the bacteria showed a central tissue that contained much lower...
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fluorescence compared to wild type (Figure 5C compared to 5G). While control M. truncatula nodules are cylindrical due to the presence of an apical persistent meristem, 3SS:AtSINAT5DN fix+nodules were round shaped indicating a precocious arrest of the meristem (Figure 5F compared to 5B). RT-PCR on root tissue of the different F1 lines showed that there was a correlation between the level of transgene expression and the phenotype (Figure 5D and E). In lines with a high expression level only fix+nodules were observed whereas in moderately expressing lines, fewer nodules were formed compared to wild type, but these were functional (Figure 5D).

Nodulation consist of rhizobial infection and nodule primordium formation, which are tightly linked in time and space but which can be studied separately. To discriminate the effect of 3SS:AtSINAT5DN transgene expression on infection or nodule primordium formation, ten plants of two different R108 3SS:AtSINAT5DN F2 lines were grown on nitrogen poor medium and inoculated with R. meliloti (pHC60-gfp). Nodule primordia and infection events, i.e. a curled root hair containing an infection thread, were counted at 10
dpi, when an incipient nodule primordium is protruding out of the cortex, at 16 dpi, when a young round-shaped nodule is seen in which ITs have reached the cells for bacterial uptake and nitrogen fixation is started and at 29 dpi, when an apical meristem develops and a functional nodule is formed. At 10 dpi, 35S:AtSINAT5DN lines contained fewer infection events (on average 0.3 per plant) compared to control plants (on average 2.8 per plant) (Figure 6A), and fewer nodule primordia (on average 1 np compared to 5 np per plant; Figure 6B). At 20 dpi, the number of nodule primordia on 35S:AtSINAT5DN plants was equal to the number observed on control plants (on average 12 primordia per plant). When the infection events were counted at 20 dpi, a considerably higher number was counted compared to control plants (on average 9.5 events compared to 6.8 events per plant; Figure 6A). Finally at 29 dpi, an average of 10 nodules was observed in control plants whereas only an average of only 6 nodules was counted on the transgenic lines (Figure 6B). At that stage, in control roots the number of infection events was lower compared to 16 dpi. On the contrary, the number of infections was still high in the 35S:AtSINAT5DN lines (on average 3.9 per control plant compared to 9.3 per 35S:AtSINAT5DN plant; Figure 6A).

**Microscopical analysis of 35S:AtSINAT5DN nodules**

To unravel the stages during which nodule formation is blocked in the 35S:AtSINAT5DN transgenic plants, nodules at different time-points after infection were analyzed by light microscopy. At 16 dpi, small round-shaped nodules are observed on wild type roots, with apical meristem, an infection zone and a small fixation zone (Figure 7A, B and C). Sections of 35S:AtSINAT5DN nodules at the same stage showed that the central tissue of the incipient nodule was completely disordered (Figure 7D). Infection threads had reached the primordium cells but had a thick appearance compared to wild type (Figure 7D and E compared to 7A and B, arrowheads). Some bacterial uptake inside plant cells was observed (Figure 7D, asterisks), but the zonated structure of the indeterminate nodule was lacking, no
FIGURE 7: Bright-field microscopy on 35S:AtSINAT5DN (SDN) and wild type M. truncatula nodules.

A, young wild type nodule containing a nodule meristem (m), infection zone (i) and fixation (f) zone. B, detail of infection zone of wild type young nodule. C, 25dpi wild type fixation zone with infected cells filled with bacteroids surrounding the vacuole. D, young SDN nodule showing a disordered structure and few infected cells (asterisks). E, detail of infected region in D, thicker ITs (arrowheads) appear and cells show signs of degradation (arrows). F, wild type M. truncatula 61dpi senescent nodule tissue. Arrows indicate cells with senescent features. G, young SDN nodule slightly more infected (asterisks) than the 35S:AtSINAT5DN nodule in D. H, detail of G. I, detail of infected degrading cells in the SDN nodule shown in J. J, 25dpi SDN nodule showing a small fixation zone (f), showing cell degradation and senescence (arrows). K, 25dpi SDN long nodule without meristem, showing infected cells and early senescence of the nodule central tissue. L, detail of K. Arrowheads, abnormal infection threads; arrows, signs of cell degradation and senescence; asterisks, infected cells containing bacteria.
meristem was observed and there was no formation of a fixation zone (Figure 7D compared to A). Moreover, cell wall disruption was often observed (Figure 7E, arrows). In some lines, the development of a fixation zone had proceeded further (Figure 7G). Also in these lines, the infection threads showed an irregular structure compared to the ITs in wild type nodules and no nodule meristem could be detected (Figure 7G and H). However, a slightly higher number of infected cells filled with bacteria could be seen. A close-up of this region in Figure 7H showed that although the plant cells looked like filled with bacteria, they showed signs of bacteroid degradation, which normally occurs at the onset of senescence during wild type nodulation (Figure 7H compared to C and F). In still other lines, although no meristem was detected, some infected cells with a healthy appearance were observed, resulting in a small fixation zone (Figure 7I and J), but the symbiotic interaction was quickly lost as this zone was followed by a region of senescence where the bacteroids disappeared and plant cells lost their rigidity (Figure 7I and J). Finally, in some cases, long-shaped nodules were observed (Figure 7K), showing that the apical meristem has been active. However, sectioning through such a nodule at 26 dpi showed the lack of a nodule meristem and a central tissue that was senescing (Figure 7K and L). Electron microscopical analysis on young and mature wild type and 35S:AtSINAT5DN nodule tissue revealed that the infection thread matrix of the ITs formed in 35S:AtSINAT5DN lines was more dense compared to wild type IT matrix as seen by the dark staining (Figure 8A compared to B). The bacteria within the IT looked healthy, although fewer bacteria and more matrix were observed (Figure 8A). Once the infection threads reached the nodule primordium, bacterial uptake was taking place (Figure 8D and E). The infection droplets, by which bacterial uptake occurs, looked normal (Figure 8D and E compared to C), but after bacterial uptake in the cytosol of the plant cells, the development of the symbiosome was hampered (Figure 8F). Whereas normally the symbiosome membrane tightly encloses a single bacteroid (Figure 8G and H), in the transgenic nodules, the symbiosome membrane only loosely surrounded the bacteroid and the symbiosome space was visible (Figure 8F, arrows). At later steps, symbiosome division, which constitutes a synchronous division between the membrane and the bacteroid, was hindered as large symbiosomes could be detected containing several degrading bacteroids (Figure 8I and J). Besides the bacterial degradation, also the plant cells were senescing as seen by cell wall apposition (Figure 8K, arrow) and loss of cell integrity (Figure 8L).
AtSINAT5 interacts with several classes of M. truncatula proteins.

To identify M. truncatula proteins interacting with AtSINAT5, a yeast-two-hybrid analysis (Fields and Song, 1989) was performed with AtSINAT5WT and AtSINAT5DN as baits. cDNA libraries of M. sativa roots and of M. truncatula R108 10 dpi nodules cloned in pADGAL4 were screened with the AtSINAT5WT and AtSINAT5DN fused to the GAL4 DNA-binding domain (see Methods).

Both baits were cotransformed in yeast with each cDNA library and after growth on triple selection medium (lacking histidin, tryptophan and leucine) the clones containing the cDNA’s that encode the interacting proteins were isolated (see Methods). The four different
screening combinations revealed a total of 146 clones. Elimination of false positives and household genes (see Methods) resulted in 54 clones corresponding to putative interactors. The 54 corresponding plasmids were individually retransformed in yeast together with the two bait constructs to verify the interaction. After 3 to 8 days of growth on triple selection medium, 26 interactions were confirmed (Table 2).

The prey clones were sequenced and subjected to a BlastN search using the TIGR EST dataset (www.tigr.org). The identity of the clones is given in Table 2. The in silico expression, as shown on www.tigr.org, is also summarized in Table 2. 9 clones corresponded to proteins that function in ubiquitin-mediated proteolysis of which 6 were homologous to SINA-domain containing proteins (Table 2, red functional class). Most other clones have been found back in cDNA libraries derived from nodulated roots and hence have been assigned as nodulin genes (Table 2, yellow functional class). They were mainly part of cDNA libraries from functioning nodules but were also found back in a lower amount in libraries from roots a few days after S. meliloti inoculation.

The binding affinity of the interaction was estimated by the application of a range of 3-AT concentrations in the growth medium and the maximum 3-AT concentration at which growth of each clone still occurred is indicated in Table 2. The highest binding affinity on growth medium containing 80mM 3-AT was found when yeast was cotransformed with AtSINAT5DN baits together with MtSINA2, MtSINA3, MtSINA5, or the MtSINA encoding AL377008 clone, and also with Lb1 or with MtN22 clones as preys. For the latter 2 clones, the binding affinity for AtSINAT5WT was weaker (20mM 3-AT and 5 mM 3-AT, respectively). Cotransformation of the yeast strain with the prey clone TC79966 and both AtSINAT5 clones resulted in yeast growth on 40mM 3-AT. Growth to 20mM 3-AT after cotransformation with AtSINAT5 was observed for 6 prey clones (MtSINA1, MtSINA4, TC76877, TC86088, TC101807 and TC80915). Six other prey clones (TC76365, TC95054, TC85422, TC86333, TC88855 and TC87254) grew on 10mM 3-AT after cotransformation with the AtSINAT5 baits. Five prey clones (TC80418, TC106577, TC97283, TC78450 and TC85903) only showed growth to 5mM 3-AT, and the remaining two prey clones, TC106310 and TC85831, grew on histidin lacking triple selection medium, but not when 3-AT was supplied.

In summary, the yeast-two-hybrid analysis using the A. thaliana SINAT5WT and AtSINAT5DN proteins as baits revealed interaction in yeast with 26 M. truncatula proteins, including 6 MtSINA proteins (MtSINA1 to 5 and the protein encoded by the AL377008 sequence), 3 ubiquitin-pathway related proteins, 11 nodulins, and 6 other proteins.
<table>
<thead>
<tr>
<th>TC N°</th>
<th>TIGR Annotation</th>
<th>In silico expression</th>
<th>Protein Function class</th>
<th>DN</th>
<th>WT</th>
<th>3-AT conc (mM)</th>
<th>Isolated from</th>
</tr>
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<tbody>
<tr>
<td>AL377008</td>
<td>Singleton; homologue to seven in absentia-like protein (Arabidopsis thaliana)</td>
<td>MSeo singletone</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>80</td>
<td>N/ND</td>
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<tr>
<td>TC10430</td>
<td>M1N5A6 similar to S1A1p</td>
<td>Mycorrhizal roots (0.01) and Phoma-infected leaf (0.03)</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>80</td>
<td>N/ND</td>
</tr>
<tr>
<td>TC100204</td>
<td>M1N3A3 similar to ring finger E3 ligase S1A1T5 (Arabidopsis thaliana)</td>
<td>Nematode-infected root (0.06), KVO (0.02), DSSL (0.02), GESD (0.02), Exc (0.01)</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>80</td>
<td>N/WT</td>
</tr>
<tr>
<td>TC102374</td>
<td>M1N1A1 homologue to ubiquitin ligase S1A1T5</td>
<td>Mycorrhizal roots (0.03), developing flower (0.03), DSSL (0.02), Developing root (0.03) and MUA-ac (0.01)</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>20</td>
<td>N/WT</td>
</tr>
<tr>
<td>TC102389</td>
<td>M1N2A2 similar to seven in absentia-like protein - Arabidopsis thaliana</td>
<td>Drought (0.04), exc (0.02), mycorrhizal roots (0.01), developing leaf (0.01), flower (0.02) and phosphate-starved leaf (0.01)</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>80</td>
<td>N/WT</td>
</tr>
<tr>
<td>TC102382</td>
<td>M1N2A4 similar to S1A1p</td>
<td>Drought (0.02), developing stem (0.02), MSeo (0.01), Mycorrhizal roots (0.01), MSeo (0.01) and exc (0.01)</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>x</td>
<td>20</td>
<td>N/WT</td>
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<tr>
<td>TC76665</td>
<td>Ubiquitin-conjugating enzyme</td>
<td>GVN (0.06), MSeo (0.04) among many others</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>10</td>
<td>R/WT</td>
<td></td>
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<tr>
<td>TC60654</td>
<td>Ubiquitin carrier</td>
<td>GVN (0.04) among many others</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>10</td>
<td>R/WT</td>
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<tr>
<td>TC60648</td>
<td>similar to Proteasome subunit beta type-5 precursor</td>
<td>KVO (0.03), KVO (0.02), KVO (0.04) among some others</td>
<td>Proteasome degradation pathway</td>
<td>x</td>
<td>5</td>
<td>N/WT</td>
<td></td>
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<tr>
<td>TC16687</td>
<td>Leghemonophin 2</td>
<td>GVN (0.5), MSeo (0.13), GVN (0.06), Nodulated root (0.13), MR108 (0.09)</td>
<td>Leghemonophin</td>
<td>x</td>
<td>x</td>
<td>20</td>
<td>N/WT</td>
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<tr>
<td>TC16692</td>
<td>Leghemonophin 1</td>
<td>GVN (0.2), GVN (0.25), MSeo (0.24), MSeo (0.06), MR108 (0.06), MSTW (0.71), Nodulated Root (0.29) among few others</td>
<td>Leghemonophin</td>
<td>x</td>
<td>x</td>
<td>20 with SNT, 80 with SDN</td>
<td>N/ND, R/WT</td>
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<td>TC59422</td>
<td>contains late nodule domain homologous to PeoEn3d</td>
<td>GVN (0.29), GVN (0.16), Leguminosae (0.23), Nodulated Root (0.03) MR108 (0.22)</td>
<td>late nodule</td>
<td>x</td>
<td>10</td>
<td>N/ND</td>
<td></td>
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<tr>
<td>TC105977</td>
<td>contains late nodule domain homologous to PeoEn3d</td>
<td>GVN (0.46), GVN (0.04), MSeo (0.19), MR108 (0.28)</td>
<td>late nodule</td>
<td>x</td>
<td>5</td>
<td>R/WT</td>
<td></td>
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<td>TC105978</td>
<td>contains late nodule domain homologous to PeoEn3d</td>
<td>GVN (0.05), GVN (0.04)</td>
<td>late nodule</td>
<td>x</td>
<td>5</td>
<td>R/WT</td>
<td></td>
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<td>TC105899</td>
<td>M1N22 protein precursor</td>
<td>GVN (0.45, 0.43), GVN (0.94, 0.29), MSeo (0.08), MSeo (0.01), MSeo (0.71, 0.35), Nodulated Root (0.06, 0.03), MSTW (0.24)</td>
<td>late nodule</td>
<td>x</td>
<td>5 with SNT, 80 with SDN</td>
<td>N/WT &amp; SDN</td>
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<tr>
<td>TC74549</td>
<td>Nodule-specific glycoprotein-3</td>
<td>GVN (0.05), MSeo (0.35), MR108 (0.07), GVN (0.08)</td>
<td>late nodule</td>
<td>x</td>
<td>5</td>
<td>N/WT &amp; SDN</td>
<td></td>
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<td>TC79696</td>
<td>Hypothetical protein</td>
<td>GVN (0.05), Leguminosae (0.17), GVN (0.18), MSeo (0.12)</td>
<td>late nodule</td>
<td>x</td>
<td>40</td>
<td>N/ND</td>
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<tr>
<td>TC66668</td>
<td>Contmu8-like protein 1</td>
<td>GVN (0.17), MSeo (0.18), GVN (0.09), Nodulated Root (0.02)</td>
<td>late nodule</td>
<td>x</td>
<td>20</td>
<td>R/ND</td>
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<td>TC103071</td>
<td>Contmu8-like protein 3/2</td>
<td>GVN (0.08, 0.11), GVN (0.08, 0.09), MSeo (0.12, 0.24), MR108 (0.22)</td>
<td>late nodule</td>
<td>x</td>
<td>20</td>
<td>R/WT</td>
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<td>BE992736/TC186333</td>
<td>weakly similar to Putative senescence-associated noduline protein</td>
<td>GVN, singletone GVN (0.16), GVN (0.11) among few others</td>
<td>late nodule</td>
<td>x</td>
<td>10</td>
<td>R/ND</td>
<td></td>
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<tr>
<td>AL365588/TC305915</td>
<td>similar to NADH dehydrogenase (ubiquinone) chain 5</td>
<td>singletone in MSeo (0.08) among few others</td>
<td>late nodule</td>
<td>x</td>
<td>20</td>
<td>N/WT</td>
<td></td>
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<td>TC86803</td>
<td>Hypothetical protein</td>
<td>KVO (0.11), Nodulated Root (0.09), KVO (0.22), among many others</td>
<td>late nodule</td>
<td>x</td>
<td>5</td>
<td>N/WT</td>
<td></td>
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<td>TC166501</td>
<td>14_3_2-like protein</td>
<td>MSeo (0.33), GVN (0.2), GVN (0.15), KVO (0.22), GVN (0.15), Nodulated Root (0.09), MR108 (0.45)</td>
<td>MR108 (0.04) among many others</td>
<td>x</td>
<td>0</td>
<td>N/WT</td>
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<tr>
<td>TC87154</td>
<td>similar to Vesicle-associated Protein, VAP27 (62%)</td>
<td>MSeo (0.03), KVO (0.04) among many others</td>
<td>late nodule</td>
<td>x</td>
<td>10</td>
<td>N/WT</td>
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<tr>
<td>TC65381</td>
<td>similar to Hidrolase-containing phosphotransfer protein 1 (92%)</td>
<td>MSeo (0.15) among many others</td>
<td>late nodule</td>
<td>x</td>
<td>0</td>
<td>R/WT</td>
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<td>TC85655</td>
<td>Calponin</td>
<td>No nodule-related libraries</td>
<td>late nodule</td>
<td>x</td>
<td>10</td>
<td>N/WT</td>
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</table>

**TABLE 2:** M. truncatula TC sequences which code for proteins that interact with ASINAT5 in the yeast two-hybrid analysis. TC number, TIGR annotation, in silico expression pattern, protein function class, interaction with ASINAT5DN (DN) or ASINATSWT (WT), maximal 3-AT concentration that allows growth on triple selection medium, and the screening from which the clone was isolated are given in the different columns. N, 10 dpi Nodule M. truncatula cDNA library; R, M. sativa root cDNA library; The full in silico expression is given for the identified M1N A protein encoding TC’s (blue), whereas for the nodulins (yellow) and the other clones isolated from nodule and non-nodule libraries (blue) only the nodulation cDNA libraries are given. Between brackets the expression is shown as the percentage of the ESTs in a certain cDNA library. Abbreviations of the cDNA libraries listed: KV3, seedling roots 3dpi; DSSL: leaves infected with Colletotrichum trifoli; GESD: early seed development; Ecc, elicited cell-culture; Miba, N-starved root tips; MBC, 3 weeks pi mycorrhizal roots; GVN, 1 month old nodules; MSeo, 4dpi roots; GVN, 40dpi semenscences nodules; MR108, developing young nodules; MSeo, root nodules 4 and 10 dpi; MSTW, 17 and 20day old whole roots at 3 and 6dpi; KVO, seedling roots 1dpi; KVO, seedling roots 2dpi; KVO, seedling roots just prior to S. meliloti inoculation.
To screen for the potential in vivo interaction of AtSINAT5 with M. truncatula proteins isolated by yeast two-hybrid analysis, co-immunoprecipitation of the proteins was performed using A. thaliana cell suspension transformation (Kiegerl et al., 2000, see Methods). The AtSINAT5DN sequence was fused to 3 hemaglutinin (3-HA) epitope tags and the MtSINA clones were fused to a 3 MYC-epitope tag. The clones were co-transfected in A. thaliana protoplasts (see Methods). The detection of the transiently expressed fusion proteins through western analysis using HA- and MYC-tag specific antibodies revealed that only for MYC-MtSINA1 and MYC-MtSINA3 a desirable amount of protein was obtained to analyze the interaction with the HA-AtSINAT5DN fusion protein. Co-immunoprecipitation using Protein G beads to precipitate the anti-HA/ HA-AtSINAT5DN complex was performed on protein extracts of protoplasts co-expressing the MYC-MtSINA1 or MYC-MtSINA3 fusion proteins with the HA-AtSINAT5DN protein, and on protein extracts only expressing the MYC-MtSINA1 or MYC-MtSINA3 protein (as a negative control). As shown in Figure 9, SDS-PAGE and subsequent immunoblotting, of input and immuno-precipitated (IP) fractions demonstrated expression of the MYC-fusion proteins in the input fractions (Figure 9, upper lanes), using an anti-cMYC specific antibody. Immunoblotting of the IP’s is shown in the lower lanes of Figure 9. Negative control IP’s on extracts containing only the MYC fusions do not show signal (Figure 9, lane 1 and 3, lower part), whereas the co-IP’s on protein extracts expressing HA-AtSINAT5DN and MYC-MtSINA3 or MYC-MtSINA1 (Figure 9, lane 2 and 4, lower part) revealed a faint band of 33kD, which is slightly larger than the detected light chain of the HA-antibody, indicating an interaction of both MYC-MtSINA fusion proteins with HA-AtSINAT5DN.

![Figure 9: Immunoblots were done using the anti-cMYC antibody for detection of the MYC-MtSINA fusion proteins after co-immunoprecipitation with anti-HA/ HA-AtSINAT5DN proteins. Input fractions (upper part) contain 1/10 of the amount of protein extract used for co-immunoprecipitation. The protein extracts were prepared from protoplasts transfected with pRTMYC-MtSINA3 (lane 1) or pRTMYC-MtSINA1 (lane 3) as negative controls and cotransfected with MYC-MtSINA3 or MYC-MtSINA1 and HA-AtSINAT5DN (lane 2 and 4, resp.). The molecular weight of the MYC-MtSINA3 and MYC-MtSINA1 proteins is approximately 33kD. Negative control immunoprecipitations (IP) on extracts containing only the MYC fusions do not show signal (lane 1 and 3, below), whereas the IPs on the extracts with co-expression of both tagged proteins show a faint signal of 33kD, just above the light-chain of the HA-antibody of 25kD (lane 2 and 4 of under part).](image-url)
Gene expression profiles for the interacting proteins of AtSINAT5 in M. truncatula roots and nodules

Nodulation of 35S:AtSINAT5DN roots resulted in two defects. The infection was hampered because abnormal infection threads were formed and after bacterial uptake symbiosome formation was impaired. To gain insight into which process the potential AtSINAT5DN and AtSINAT5 interacting proteins are active, qRT-PCR analysis was performed to study the temporal expression pattern of the genes encoding the putative interacting proteins.

Nodule tissue was harvested from M. truncatula Jemalong J5 plants grown in perlite under nitrogen starvation and inoculated with S. meliloti 1021 pH60(GFP). The harvested plant material included zone I regions of uninoculated roots, i.e. of roots at the zone of emerging root hairs susceptible for rhizobial infection, of two consecutive stages of infection thread growth and primordium development (6dpi and 9dpi), 16dpi young nodules, 40dpi mature elongated nodules, and 48dpi senescing nodules. In addition, the 40dpi nodules were sectioned resulting in an apical nodule (AN) part and proximal nodule (PN) part. The AN tissue contained the meristem, the infection zone and a minor part of the fixation zone, and the proximal nodule sample consisted of the major fixation zone and a senescent zone at the base of the nodule.

The genes coding for MtSINA proteins showed differential expression patterns. The transcript level of 2 clones, MtSINA2 and MtSINA3, was increased at 6 and 9dpi compared to the level in control roots. Next, the transcript level diminished and then raised again in mature and senescing nodules. MtSINA3 expression in mature nodules was also more abundant in the PN sample, which contained the fixation zone and a small senescing zone (Figure 10 Group 1).

Three other clones, MtSINA1, MtSINA5 and the MtSINA encoded by the singleton AL377008, had a background transcript level in all tissues analyzed but were significantly upregulated in the proximal part of 40 dpi nodules (Figure 10 Group 2). The MtSINA4 transcript level was already higher at 6dpi compared to the level in control roots and continuously increased during the nodulation process. In mature nodules, transcripts were mainly found in the proximal fixation zone (200 fold higher compared to control roots; Figure 10 Group 3).

Together, these experiments show that there are mainly three stages during which MtSINA proteins are expressed, early at the onset of the nodulation process and later during nitrogen fixation and senescence.
The other genes from which the proteins are involved in ubiquitin-mediated protein degradation were not highly upregulated in nodulation. Still, a small raise in transcript level could be seen early (6 and 9dpi) and late (40dpi PN or senescence) for TC76365 and TC80418 (Figure 10 Group 1) and in the proximal fixation zone (PN) for T95054 (Figure 10 Group 2).

The same pattern as for MtSINA4, which displays a highly upregulated transcript level at 6dpi that continuously increased during the nodulation process and dropped at nodule senescence, was observed for clone TC100596, coding for MtN22, TC106577, encoding a PsENOD3-like domain protein, TC76877, encoding Lb2, and although with an increased transcript level of only 2 to 4 fold, clone TC106310, coding for a 14_3_3 protein (Figure 10 Group 3).

A very clear expression pattern, including a transcript level increase in nodule primordia (9dpi) which reached a maximum in fixing nodules (16dpi and 40 dpi) but not in senescing nodules, was observed for TC107926, TC86088, TC78450, TC85422, and TC97283 (Figure 10 Group 4). Also the clones TC86333, TC79966, showed this pattern, with an increased transcript level from 16dpi on (Figure 10 Group 4). In the sectioned nodules, transcripts were about equally present in the apical and proximal region, indicating that the expression is correlated to the fixation zone. The transcript level of two clones, TC80915 and TC85903, showed an increase in fixing nodules, and more or less stayed at this level in senescent tissue (40dpi and Sen; Figure 10 Group 5).

The transcript level of 4 clones (TC88855, TC85831, TC87254, and TC106592) did not significantly rise during nodule formation but was constitutive in all samples analyzed (Figure 10 Group 6). However, the transcript level of 2 clones, TC85831 and TC87254, dropped 2 to 3 times at 9dpi.
SINA proteins in Medicago truncatula

FIGURE 10: qRT-PCR expression analysis of M. truncatula genes isolated by the yeast two-hybrid analysis on M. truncatula Jemalong roots and different nodulation stages. The values of the relative expression changes as compared to the level detected in uninoculated nitrogen-starved roots are shown. NI-root, uninoculated nitrogen-starved root zone I region; 6dpi, root tissue containing infection threads after 6 days of S. meliloti inoculation; 9dpi, nodule primordium tissue; 16dpi Ynod, young nodules 16 days after S. meliloti inoculation; 40dpi Fix, fixing nodules with meristem, infection, fixation and early senescence zone; 40dpi AN, apical part of 40 dpi nodule containing the meristem, the infection zone and the early fixation zone; 40dpi PN, the proximal part of 40 dpi nodule containing the majority of the fixation zone and the starting senescence zone at the nodule base; 48dpi sen, 48dpi totally senescent nodules. Error bars, standard deviations on 3 technical replicates.
FIGURE 10 continued: qRT-PCR expression analysis of M. truncatula genes isolated by the yeast two-hybrid analysis on M. truncatula Jemalong roots and different nodulation stages. The values of the relative expression changes as compared to the level detected in uninoculated nitrogen-starved roots are shown. NI-root, uninoculated nitrogen-starved root zone I region; 6dpi, root tissue containing infection threads after 6 days of S. meliloti inoculation; 9dpi, nodule primordium tissue; 16dpi Ynod, young nodules 16 days after S. meliloti inoculation; 40dpi Fix, fixing nodules with meristem, infection, fixation and early senescent zone; 40dpi AN, apical part of 40dpi nodule containing the meristem, the infection zone and the early fixation zone; 40dpi PN, the proximal part of 40dpi nodule containing the majority of the fixation zone and the beginning senescence zone at the nodule base; 48dpi sen, 48dpi totally senescent nodules. Error bars, standard deviations on 3 technical replicates.
DISCUSSION

Nodule formation is a tightly regulated process that not only involves specific signal exchange between the symbionts but also requires the coordinated activation of developmental processes to allow bacterial invasion, the induction of cell division and proper continuation of nodule development. Ubiquitin-mediated proteolysis is a common regulatory pathway and presumably involved in nodulation. Moreover, nodule senescence is characterized by massive protein degradation.

E3 ligases are the components of the ubiquitin-mediated degradation machinery that specifically select the target proteins for degradation. To day, only one E3 ligase has been described to play an important role in nodule development. The APC^{CCS52A} complex degrades mitotic cyclins in the infected cells which induces endoreduplication resulting in polyploidy needed to assure cell enlargement to host the vast amount of nitrogen fixing bacteroids (Vinardell et al., 2003; Kondorosi et al., 2005).

Here we report on the role during nodule development of another family of E3 ligases, the SINA proteins. We provide evidence that they are important for infection thread growth, during symbiosome differentiation and in nodule senescence.

The only characterized member of the SINA proteins in plants is the AtSINAT5 from Arabidopsis thaliana. The protein is involved in lateral root formation as ectopic expression of AtSINAT5 results in fewer lateral roots whereas ectopic expression of a dominant negative form produces more lateral roots (Xie et al., 2002). The dominant negative protein has an amino acid substitution in the RING domain resulting in the loss of ubiquitin ligase activity, while the binding with the target AtNAC1 was intact (Xie et al., 2002). Upon heterodimerization, the activity of the interacting wild type SINAT5 protein is inhibited (Xie et al., 2002).

TBlastX analysis using the AtSINAT5 sequence on the M. truncatula EST datasets (www.tigr.org; Journet et al., 2002) resulted in the identification of 6 Tentative Consensus sequences clones and 3 singleton ESTs that might encode SINA proteins. The in silico northerns did not reveal any clone that was clearly upregulated during nodulation. Consequently, we ectopically expressed AtSINAT5 and the dominant-negative form AtSINAT5DN in M. truncatula R108 and Jemalong plants to unravel a potential role of SINA-proteins in nodulation.

Transgenic M.truncatula lines ectopically expressing AtSINAT5 or AtSINAT5DN revealed several features that remind of or are similar to the A. thaliana phenotypes. The
35S:AtSINAT5DN plants showed a more vigorous growth and larger leaves compared to M. truncatula control plants. This phenotype has not been described in Arabidopsis but was reported for the transgenic plants that ectopically express AtNAC1, which codes for a NAC transcription factor that is a specific target of AtSINAT5 (Xie et al., 2000; Xie et al., 2002). Also the number of lateral roots was modulated by introducing AtSINAT5 and AtSINAT5DN in M. truncatula, similar to what was observed for A. thaliana. 35S:AtSINAT5 plants produced fewer lateral roots per cm and 35S:AtSINAT5DN plants contained more lateral roots per cm. The effect of lateral root formation was even more pronounced when roots were treated with auxin to induce lateral root formation. This experiment also indicated that it was not the increased shoot growth that was responsible for the increase in lateral root number but that the effect was due to a direct involvement of SINA proteins in the process.

Together, these experiments demonstrate that AtSINAT5 can target M. truncatula proteins for degradation and that AtSINAT5DN is able to inhibit endogenous SINA proteins. The M. truncatula orthologue of AtSINAT5 is so far not known although MtSINA1 is a good candidate. The clone has been found in cDNA libraries from developing roots and the deduced amino acid sequence showed the highest similarity to AtSINAT5.

Nodulation on the 35S:AtSINAT5 expressing M. truncatula transgenic lines was not different compared to the control lines indicating that AtSINAT5 does not ubiquinate M. truncatula targets that would be involved in nodule formation and that the AtSINAT5 orthologue of M. truncatula is not involved in nodulation.

On the other hand, ectopic expression of AtSINAT5DN had a drastic effect on nodule formation. Nodule primordium formation was not hampered but bacterial infection was delayed. Infection threads were formed but the infection was not proceeding well resulting in an abundant initiation of new infections at later time points. This is a typical observation for mutants with a defected IT growth as the mechanism that controls the number of infection events is not switched on (Tsyganov et al., 2002; Tansengco et al., 2003; Veereslingham et al., 2004). Light and electron microscopy confirmed that the ITs were deformed. They were broader compared to wild type and contained a denser matrix with fewer bacteria. Eventually some ITs did reach the nodule primordium and penetrated between the primordium cells. However, also the formation of symbiosomes was affected. The bacterial uptake, via unwalled infection droplets did occur but the freshly formed symbiosomes did not look normal. In wild type plants, the symbiosome membrane closely surrounds the bacteroid, but in 35S:AtSINAT5DN nodules there was a broad space between the bacteroid and the symbiosome membrane. Large symbiosomes were seen in which
several degenerating bacteroids reside resulting in early nodule senescence. As a consequence, the nodule did not develop further; no meristem was installed resulting in small, round fix nodules.

During nodule formation, the bacterial infection is controlled by the plant and impaired infection often results in the elicitation of defense responses to contain the infection (Banba et al., 2001; Novak et al., 2004). Clearly, also the impaired infection threads elicited defense reactions as often cell wall appositions and loss of cell integrity was observed in the defective nodules.

Together, analysis of the 35S:AtSINAT5DN nodulation indicated that SINA proteins and hence targeted protein degradation are involved at two steps during nodule formation, during IT growth and during symbiosome formation (Figure 11).

Yeast two-hybrid analysis using AtSINAT5 and AtSINAT5DN as baits resulted in the isolation of 6 clones encoding putative M. truncatula SINA proteins. A strong binding affinity (growth to 80mM 3-AT) was found for all MtSINA’s, except for MtSINA1 and MtSINA4 which only grew up to 20mM 3-AT and of which MtSINA4 was shown to interact only with the AtSINAT5 wild type protein. The weak interaction did reflect true interactions as shown by co-immunoprecipitation between AtSINAT5DN and resp. MtSINA1 and MtSINA3.

Q-PCR analysis on RNA derived from developing nodules provided a hint about which endogenous SINA proteins could be inhibited by AtSINAT5DN dimerization. MtSINA2 and MtSINA3 transcripts were accumulating at 6 and 9 dpi, the stage at which ITs are growing towards the nodule primordia. Therefore, it is highly probable that AtSINAT5DN is inhibiting those MtSINA proteins thereby hampering their ligase activity (Figure 11). The transcript level of MtSINA3 and MtSINA4 and to a lesser degree MtSINA1 and MtSINA5 was elevated in the fixation zone. Hence, inactivation of the corresponding proteins by AtSINAT5DN dimerization might be the underlying reason of the symbiosome defect (Figure 11). Interestingly, a third expression pattern was found in senescing tissue. All 6 MtSINA transcripts were more abundant in 48dpi senescing nodules, which were green instead of pink, indicating the lack of a healthy fixation zone. Thus, besides a role for MtSINA proteins in infection and symbiosome formation, they might be involved during the senescence process (Figure 11). The involvement in senescence could not be detected by analyzing the transgenic plants, probably because of the pleiotrophic phenotype caused by ectopic AtSINAT5DN expression and the precocious inhibition of symbiosome formation. Also other clones coding for different subunits of the ubiquitin-mediated proteolysis were identified by the two-hybrid screen, and encoded proteins homologous to polyubiquitin, ubiquitin carrier 4
and to ubiquitin E2-conjugating enzymes, which presumably contribute to the proteasomal degradation process acting during nodulation. Indeed, the transcript level of the corresponding genes was upregulated at one of the three stages during nodulation or was constitutive throughout the nodule developmental process. Also potential targets for the endogenous SINA proteins were identified in the yeast two-hybrid experiment. Indeed, it has been shown that RING proteins can bind the targets of homologous members but are not able to perform the ubiquitination reaction (Joazeiro and Weisman, 2000). The interaction of AtSINAT5 and AtSINAT5DN with the potential targets was weak as the majority of the interaction resulted in yeast growth on less than 40 mM 3-AT, an observation expectable for heterologous binding. As in 35S:SINAT5WT plants, no nodule related phenotype was observed, it is indeed very plausible that the protein could weakly and reversibly bind the targets but not perform the ligation reaction.

q-PCR analysis of the 17 potential target clones revealed that the level of leghemoglobin 2, MtN22, one protein homologous to PsENOD3 and one protein homologous to 14_3_3 proteins might be controlled by ubiquitination through MtSINA2 and MtSINA3 at the onset of nodulation as the transcript level of the corresponding genes rise at the onset of nodulation. The expression of Lb2 is intriguing: the gene is switched on at stages earlier than nitrogen fixation, the time points at which leghemoglobin controls the oxygen level in the infected cells (Sharifi, 1983; Ott et al., 2005). Also in S. rostrata, a leghemoglobin encoding gene has been found the expression of which was correlated with infection thread growth before the onset of nitrogen fixation (J. Den Herder et al., submitted). It will be

**FIGURE 11**: Proposed model for MtSINA regulation of nodulation in *M. truncatula*.

The different stages of nodulation are shown by the drawings and specific responses during each stage are written down. Grey boxes indicate the three stages at which MtSINA target genes are expressed. Blue boxes indicate the MtSINA proteins action points, MtSINA2 and MtSINA3 are upregulated at the early stages, probably to target the early interacting nodulins. MtSINA3, is also upregulated at late stages of nodule functioning, probably for targeting of the late nodulin interacting proteins. MtSINA4 shows a gradual increase throughout nodulation. All MtSINA’s are expressed at the trigger of nodule senescence. NP, nodule primordium; orange star, rhizobia; green block, NF molecules.
interesting to study the localized expression pattern of MtLb2. However, no target genes were identified for which the expression level drops again after the early nodulation stages, which might indicate that the early targets for nodulation were not isolated in the yeast two-hybrid analysis.

The majority of the clones had an expression pattern correlating to the functional nodules in which fixation is taking place. The corresponding proteins might be involved in symbiosome formation and hence might be potential targets of MtSINA3 or MtSINA4 or could be degraded at the onset of senescence (Figure 11). For instance, CaM-like proteins were localized in the symbiosome space (Liu et al., 2006). Leghemoglobinbs might be degraded at the onset of senescence.

In summary, our data provide a sound basis to study the role of targeted protein degradation in nodule development in M. truncatula. The obvious functions to investigate are the MtSINA genes that were identified and that are good candidates to control sub-programs of nodule development by targeting specific nodulin proteins for proteolytic degradation.

METHODS

Plant material, bacterial strains, and growth conditions.

Growth of Medicago truncatula R108 and Jemalong J5, Sinorhizobium meliloti 1021 and plant inoculation were as described in Mergaert et al. (2003) or at www.isv.cnrs-gif.fr/embo2/manuels/index.html. S. meliloti 1021 and Sm1021 (pHC60-GFP) (Cheng and Walker, 1998) were grown at 28°C in YEB (Vervliet et al. 1975), which was supplemented with 10 mg/l tetracycline for the pHC60-GFP strain.

In vitro growth occurred in square petri dishes (12 × 12 cm) on Kalys agar (HP 696-7470 Kalys, France) containing SOLi medium (as described at www.isv.cnrs-gif.fr/embo2/manuels/index.html) supplemented with 1 mM NH₄NO₃ and for 35S:AtSINAT5 and 35S:AtSINAT5DN lines with glufosinate-ammonium (basta; Pestanal Sigma-Aldrich Riedel-de Haen), at 25°C in a 16h photoperiod and a light intensity of 70 µE per s m⁻².

Lateral roots (LR) were counted and the main root length was measured to determine the amount of LRs/cm after 20 or 40 days of growth for 35S:AtSINAT5DN and 35:AtSINAT5WT lines, respectively. In vitro grown 6-day-old 35S:AtSINAT5DN lines and wild type R108 and J5 plants were transferred to new plates containing 2 µM α-naphtalene acetic
acid (NAA, DUCHEFA Biochemie, Haarlem, NL) for another 6 days, after which lateral roots were counted and the main root length was measured to determine the amount of LRs/cm.

For nodulation experiments, 35S:AtSINAT5WT and 35S:AtSINAT5DN lines were selected by 2 weeks of in vitro growth on plates with medium as described above supplemented with basta, subsequently transferred to perlite and inoculated with S. meliloti. Analysis of nodule morphology and harvesting for light and electron microscopy was performed on F1 and F2 plants at 10dpi, 16dpi, 20dpi and 29dpi or, when only a single time-point was analyzed, at 22 dpi.

For qRT-PCR analysis, wild type M. truncatula J5 plants were grown for 7 days in perlite and inoculated with Sm1021 (pHC60-GFP). A total of 30 plants was used for harvesting tissue at all nodulation stages: the zone I region of uninoculated roots, of 6dpi IT-containing roots, of 9 dpi primordium-containing roots, and also young 16 dpi nodules, mature fixing 40 dpi nodules, and senescent 48 dpi nodules. In addition, sectioning of 40dpi nodules of 70 plants was done by separating the apical and proximal nodule parts, which contain either the meristem, infection zone and early fixation zone, or the majority of the fixation zone and senescence zone, respectively. The presence of GFP bacteria was screened for under a stereo microscope MZFLII (Leica, Wetzler, Germany) equipped with a blue-light source and a Leica GFP Plus filter set ($\lambda_{\text{ex}} = 480/40; \lambda_{\text{em}} = 510\text{nm LP barrier filter}$).

**Agrobacterium tumefaciens-mediated transformation of M. truncatula**

M. truncatula Jemalong J5 and R108 young leaves from 4 week-old plants were used for Agrobacterium tumefaciens-mediated transformation which was performed as described at [www.isv.cnrs-gif.fr/embo2/manuels/index.html](http://www.isv.cnrs-gif.fr/embo2/manuels/index.html). Binary vectors pBA002(35S:AtSINAT5WT) and pBA002(35S:AtSINAT5DN) (kindly provided by N-H Chua, Rockefeller University, New York) were electroporated in A. tumefaciens AGL0 and a single colony was isolated after selection on YEB supplemented with 100mg/L rifampicin and 100mg/L spectinomycin. After plant transformation and cocultivation, transgenic tissue was selected on the appropriate medium ([www.isv.cnrs-gif.fr/embo2/manuels/index.html](http://www.isv.cnrs-gif.fr/embo2/manuels/index.html)) containing 3mg/L basta. In vitro regenerated transgenic shoots were further grown on plates for 1 month and then transferred to vermiculite in the growth chamber. Seed pods were harvested and F1 seedlings were selected on nitrogen-rich medium supplemented with 3mg/L basta (see above) to segregate the transgenics and to obtain seed for the F2 generation.
In silico sequence identification

TBlastX analysis with the AtSINAT5 coding sequence (Genbank Acc. N° AF480944) was performed on the M. truncatula Gene Index database (MTGI release 8.0; www.tigr.org/tdb/tgi). Sequences retrieved from the yeast two-hybrid screen were in silico hybridised to the MTGI database by TBlastN analysis. DNA sequence data were assembled and further analyzed using the GCG package (Genetics Computer Group, Madison, WI), with the GAP program to determine percentages of identity and similarity between amino acid sequences, and by ClustalW analysis to attain alignments.

Statistical analysis of lateral root and nodule numbers

Lateral roots were counted and the length of the main root was measured (0.5 cm accuracy). For each line, the amount of LRs/cm was calculated for n plants. Corresponding standard errors were calculated as the standard deviation divided by the square root of n plants. Statistical analysis was performed by a two-tailed t-test on wild type versus transgenic populations.

Two experiments were performed on a total of 10 R108 35S:AtSINAT5WT lines versus wild type R108 after 40 days of growth. 10 plants per line were analyzed, resulting in a total of n=40 wild type and n=100 35S:AtSINAT5WT plants showing an average of 2.75 ± 0.29 LRs/cm and 1.33 ± 0.1 LRs/cm, respectively. T-test resulted in a P-value of 7.18E-12 <0.001, indicating a significant difference.

4 R108 35S:AtSINAT5DN lines versus a control population (M. truncatula R108 transformed with a T-DNA containing a GUS marker gene) were analyzed after 20 days of growth. 5 plants per line were analyzed, resulting in a total of n=5 wild type and n=20 35S:AtSINAT5DN plants showing an average of 2.16 ± 0.5 LRs/cm and 4.12 ± 0.57 LRs/cm, respectively (Figure 4D). T-test resulted in a P-value of 0.0257 < 0.05, indicating a significant difference.

5 R108 35S:AtSINAT5DN lines versus R108 wild type were analyzed after 6 days of growth followed by 6 days of auxin treatment (see above). 10 plants per line were analyzed, resulting in a total of n=10 wild type and n=50 35S:AtSINAT5DN plants showing an average of 1.35 ± 0.29 LRs/cm and 4.95 ± 0.33 LRs/cm, respectively (Figure 4E). T-test gave P-value of 1.65E-08 <0.001, showing a significant difference.

Nodules were counted as well and showed an average of 8.53 ± 1.02 for wild type plants (n=19), whereas an average amount of nodules of 4.22 ± 0.29 for R108 35S:AtSINAT5DN plants (n=51) was obtained (Figure 5). A t-test resulted in a P-value of
0.000519 < 0.001, showing that the number of nodules in the 2 populations is significantly different.

**Microscopy**

Bright-field microscopy was performed as described in D’Haeze et al. (1998). Nodules at 16 dpi and 25 dpi were fixed in 2.5% gluteraldehyde in 0.05 M cacodylate buffer, washed, dehydrated and embedded in Technovit 7100 (Kulzer Histo-Technik, Wehrheim, Germany) according to the manufacturer’s instructions. Sections of 5 μm were cut on a microtome (Reichert-Jung, Nussloch, Germany), mounted on Vectabond-coated slides (Sigma-Aldrich, St. Louis, MO), stained with 0.5 % toluidine blue, mounted with Depex (Sigma-Aldrich), and examined under a bright-field microscope (Leica DMBL). Images were taken with an Axiocam digital camera (Zeiss) and processed with corresponding software (Axiovision). For electron microscopy analysis, nodules were cut longitudinally and fixed in 2.5 % formaldehyde/ 3 % gluteraldehyde in 0.1 M cacodylate buffer, washed, dehydrated, and embedded in LR White hard grade (London Resin, Basingstoke, UK). Serial sections were made with an Ultracut microtome (Reichert-Jung) equipped with a diamond knife and collected on collodion-coated Cu grids, stained with 2% uranyl acetate for 12 min and examined by transmission electron microscopy (Elmiskop 101; Siemens, Karlsruhe, Germany).

**Southern hybridisation**

*M. truncatula* genomic DNA was extracted from young leaves of wild type and 35S:AtSINAT5WT and 35S:AtSINAT5DN R108 and J5 lines using the Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer’s instructions. 10 μg DNA was digested with XbaI and HindIII restriction enzymes according to standard protocols (Sambrook et al., 1989) and separated on a 1 % agarose gel. The DNA fragments were transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany) and hybridized non-radioactively using the DIG hybridization system (Boehringer). The DIG-labeled probe was generated using the AtSINAT5 primers (Table 3) and pBA002(35S:AtSINAT5) plasmid DNA as template. All procedures were done according to the guidelines of the manufacturer, and hybridized membranes were exposed to x-ray films and developed.
**RNA extraction and RT-PCR**

Total RNA was extracted from young leaves or roots of *M. truncatula* wild type, 35S:AtSINAT5WT and 35S:AtSINAT5DN R108 or J5 lines. Harvested tissue was first homogenized in TRIzol Reagent (GIBCO BRL, Life Technologies) and phase-separated with chloroform, after which the RNA was precipitated with isopropanol and resuspended in RNAse-free water. Subsequently, first-strand cDNA was synthesized from 2 μg of total RNA using the Superscript RT II kit (Invitrogen, Carlsbad, CA) (and oligo(dT)18 primer) according to the manufacturer’s instructions. Reverse transcription (RT)-PCR analysis was performed as described by Corich et al. (1998). Amplification products were detected by autoradiography after blotting to a Hybond-N nylon membrane (Amersham Biosciences) as in Corich et al. (1998). The probes were generated using AtSINAT5 or ELF1alpha primers (Table 3) and pBA002(35S:AtSINAT5) plasmid DNA or *M. truncatula* cDNA as template sequence, respectively. Probes were generated from the purified PCR product with the Rediprime II Random Prime Labelling System (Amersham Biosciences) and membranes analyzed with a PhosphorImager (Amersham Biosciences). Results were quantified to the constitutive control with ImageQuant software.

**Quantitative Reverse-Transcriptase PCR (qRT-PCR)**

Primers used for qRT-PCR were designed by using the Beacon Designer 4 Program (Biorad) and are shown in Table 3. Total RNA was isolated with the RNeasy kit as described in the manual (Qiagen, Hilden, Germany) and first-strand cDNA synthesis and quantitative PCR were done as described in Vlieghe et al. (2005). The relative expression was normalized against the constitutively expressed Translation Elongation factor 1-alpha using the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001). Reactions were done in triplicate and averaged. Primers used (see Table) had a calculated melting temperature of 59°C ± 2 and were unique in the MTGI version 8.0 (TIGR) and the Medicago EST Navigation System database (Journet et al., 2002), which was confirmed by sequencing of the amplification products.

*MtENOD40* and cystein protease (TC85913) gene-specific primers (Table 3) were used for qRT-PCR to control the samples of early nodulation stages and senescent nodulation stages (Van de Velde et al., 2006), respectively. ENOD40 expression was 15 times upregulated at 6 dpi compared to uninoculated roots, showed a 34 times upregulation at 16dpi and the highest level was observed in the apical nodule (AN) sample (40 fold), whereas in fixing nodules, proximal nodule (PN) and senescent samples the expression had decreased (data not shown). Expression of the cystein protease gene was observed from 16
dpi on, showed a lower expression level in the AN sample, and was strongly upregulated in the PN sample.

**Yeast Two-Hybrid Techniques**

The two-hybrid libraries were obtained by cloning the cDNA libraries derived from M. sativa root tissue and M. truncatula R108 10 dpi nodules in the pADGAL4 yeast two-hybrid vector (Stratagene, La Jolla, CA) (libraries provided by E. Kondorosi, ISV, GIF-sur-Yvette, France) and used as preys for the yeast two-hybrid screen. In vivo mass excision of the pADGAL4 phagemid vector was performed according to the manufacturer’s Hybridye protocol. The AtSINAT5 and AtSINAT5DN coding sequences were fused to the GAL4 DNA-binding domain of the pBDGAL4 vector (Stratagene), and used as baits. Auto-activation capacity was analyzed after cotransformation of bait with the empty pADGAL4 vector in the PJ694A yeast strain. Additional genes analyzed by (q)RT-PCR were those encoding the interacting proteins isolated by yeast two-hybrid analysis. A biological repeats was performed for all stages except for the 6 dpi and 9 dpi samples.

**TABLE 3: Overview of the M. truncatula TCs containing the ORFs encoding interacting proteins isolated by yeast two-hybrid analysis.** Additional genes analyzed by (q)RT-PCR were shown together with the primer sequences used. Column 1 gives the TC number. Column 2, the annotation as provided by TIGR or given in this study (for the MtSINA genes). Column 1, 2, and 3, are given for the MtSINA genes if available together with the TC length, the position of the ORF and the amino acid length. Column 3 and 4 show the forward and reverse primer used for the corresponding clone derived from yeast two-hybrid analysis. Additional genes analyzed by (q)RT-PCR were shown together with the primer sequences used. Column 1 gives the TC number. Column 2, the annotation as provided by TIGR or given in this study (for the MtSINA genes). Column 1, 2, and 3, are given for the MtSINA genes if available together with the TC length, the position of the ORF and the amino acid length. Column 3 and 4 show the forward and reverse primer used for the corresponding clone derived from yeast two-hybrid analysis.

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strain (James et al., 1996). After 8 days of growth at 30° C, the AtSINAT5WT-BDGAL4 did not show auto-activation of the GAL4 reporter system; the activation observed with the AtSINAT5DN-BDGAL4 fusion could be restricted by screening the cDNA libraries in the presence of 10mM 3-amino 1,2,4-triazole (3-AT; SIGMA; Bartel et al., 1993) to suppress leaking of the HIS3 marker. Growth of the transformants was tested on SD minimal base growth medium without leucine and tryptophane (Invitrogen). Screening of both cDNA libraries with the two baits revealed 146 interacting prey clones, which were selected after 8 days of growth on SD minimal base growth medium lacking histidin, leucin and tryptophane. Yeast colonies isolated in the screenings were individually transferred to new triple selection medium, and false positive clones were eliminated after 1 week of growth at 30° C. Clones that contained cDNA encoding household genes were not retained. 54 potentially interacting proteins remained and the corresponding cDNA containing pADGAL4 plasmids were purified and retransformed together with the AtSINAT5WT-GAL4BD and AtSINAT5DN-GAL4BD baits to confirm the first screening of the libraries.

The strength of the interactions was tested by the addition of 0, 5, 10, 20, 40 and 80 mM of 3-AT to this medium and growth was analyzed after 3 to 8 days.

**Transient expression in A. thaliana protoplasts and immunoprecipitation**

For transient expression in A. thaliana suspension cultured cells, the AtSINAT5WT and AtSINAT5DN coding sequences and interactor cDNAs were cloned into a pRT104-derived vector (Topfer et al., 1989) as N-terminal fusion with 3x hemaglutinin (HA) and 3x MYC epitopes, respectively. Protoplasts from Arabidopsis ecotype Columbia were prepared as described in Dangl et al. (1987) and cotransfected using the polyethylene glycol-mediated method (Kiegerl et al., 2000). Protein extracts were prepared in extraction buffer (25 mM Tris/HCl pH 7.6, 10mM MgCl2, 15 mM EGTA, 15 mM NaCl, 15 mM pNO2PhenylPO4, 15 mM β-glycerophosphate, 1 mM DTT, 1 mM NaF, 0.1mM Na3VO3, 0.5 mM PMSF, 10 µg/ml Leupeptine and Aprotinine, 0.1 % Tween and 10 % Glycerol) and total protein concentration was determined according to the Bradford method using the Biorad Protein Assay kit (Bio-rad) together with several concentrations of bovine serum albumine (BSA) as a standard. For immunoprecipitation, 300 µg protein extract was incubated with rat monoclonal anti-HA antibody 3F10 (Roche) and 25 µl of Protein G Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 2h at 4° C. Washing of immune complexes was done 3 times in 500 µl extraction buffer and protein complexes coupled to the anti-HA Protein G beads were dissolved in Tris-Glycine-SDS (TGS) sample buffer (Novex, San Diego, CA)
supplemented with 100mM β-mercapto-ethanol, boiled for 5min at 95°C and loaded on SDS-PAGE gels. SDS-PAGE was performed according to Laemmli (1970) with 12 % polyacrylamide-resolving gels and 5 % (v/v) polyacrylamide-stacking gels. Electrophoresis was performed in 1x TGS running buffer (Biorad). Next, proteins were transferred electrophoretically by wet blotting onto an Immobilon-p polyvinylidene difluoride membrane (Milipore, Bedford, MA). After overnight blocking at 4°C in 5 % skim milk dissolved in TBS-T buffer (100mM Tris-Cl, pH 7.5; 150mM NaCl; 0.1% Tween-20), the membrane was washed 3 times in TBS-T. The efficiency of the immunoprecipitation was verified using the monoclonal 12CA5 anti-HA mouse antibody (Roche). Myc-tagged proteins were detected with the monoclonal 9E10 anti-cMYC mouse antibody (Roche). Secondary anti-mouse-IgG antibody coupled to horse-radish peroxidase (HRP) was detected using a chemiluminescence detection kit according to the manufacturers instructions (Perkin-Elmer Life Sciences, Inc., Boston, MA).

ACKNOWLEDGMENTS

We would like to thank Prof. Nam-Hai Chua for providing the AtSINAT5 clones, Prof. Eva Kondorosi and group for providing the yeast two-hybrid libraries and technical assistance for in vivo mass excision and for co-immunoprecipitations. In the Plant systems biology department, we thank Wilson Ardilèz for sequencing, Annick De Keyser and Christa Verplancke for technical assistance, and Martine De Cock for help in preparing the manuscript. This research was supported by the Fund for Scientific Research-Flanders (‘Krediet aan Navorsers’ 1.5.088.99N and 1.5.192.01N) and the Interuniversity Poles of Attraction Programme-Belgian Science Policy (P5/13). GDH is indebted to the Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen for a predoctoral fellowship.
CHAPTER 6

NAC transcription factors involved in *Medicago truncatula* nodulation

Adapted from “The MtNAC1 transcription factor is involved in *Medicago truncatula* nodule development”
COVER: upper right, stereomicroscopical imaging of inoculated M. truncatula root showing a growing infection thread which is filled with GFP-expressing rhizobia. The IT is growing through the root hair towards the developing nodule primordium in the root cortex. Down left, M. truncatula transgenic root 16 days after S. meliloti inoculation containing young nodules with invading infection threads. The GFP-expression in the plant tissue is used as a reporter for cotransformation.
INTRODUCTION

Members of the plant-specific NAC (NAM, ATAF1,2, CUC2) transcription factor (TF) family are involved in various developmental processes, such as shoot apical meristem (SAM) formation, leaf senescence and lateral root development (Souer et al., 1996; Aida et al., 1997; Sablowski et al., 1998; Xie et al., 2000; Takada et al., 2001; He et al., 2005). They also play an important role in the capability of plants to interact with the environment, such as in responses to light and to various biotic and abiotic stresses (Souer et al., 1996; Aida et al., 1997; Sablowski et al., 1998; Xie et al., 2000; Takada et al., 2001; He et al., 2005). With more than 100 genes present in the Arabidopsis thaliana (At) genome and their abundance in a wide range of land plants, including dicots and monocots (Kusano et al., 2005), this class of TFs constitutes one of the largest families known in plants (Olsen et al., 2005).

The amino-terminal region of NAC proteins entails the highly conserved NAC domain (Aida et al., 1997) of which the structure mainly consists of a very twisted anti-parallel β-sheet sandwiched between two α-helices. This domain represents a novel unique transcription factor fold to which DNA-binding activity has been assigned (Ernst et al., 2004; Xie et al., 2000; Duval et al., 2002). Homo- and hetero-dimerization is equally mediated through the NAC domain, but can be influenced by the C-terminal region (Ernst et al., 2004; Olsen et al., 2004). NAC domains have also been implicated in interactions with viral proteins (Ren et al., 2000) and with RING finger proteins (Xie et al., 2002; Greve et al., 2003). The C-terminal region of NAC proteins displays a high degree of diversity among family members and functions in transcriptional activation (Xie et al., 2000; Duval et al., 2002). The level of NAC proteins is tightly regulated on the transcriptional, post-transcriptional, and post-translational level. Hormone-induced transcription (Xie et al., 2002), microRNA (miRNA)-mediated cleavage (Mallory et al., 2004; Jones-Rhoades and Bartel, 2004), mRNA long-distance transport via the phloem (Ruiz-Medrano et al., 1999), and ubiquitin-mediated proteolytic degradation has been described (Xie et al., 2002).

Lateral root initiation occurs in the pericycle cells adjacent to protoxylem poles and is controlled by environmental factors and phytohormone gradients (Casimiro et al., 2003; Aloni et al., 2006). Auxin plays a pivotal role in lateral root initiation in cell-cycle re-activation, primordium formation and the development of new vasculature (Malamy and Benfey, 1997). In A. thaliana, two NAC genes, AtNAC1 and AtNAC2, are involved in lateral root formation (Xie et al., 2000; He et al., 2005). The expression of both genes is controlled
by auxin and hence demonstrates that auxin controls lateral root formation by activation of specific sets of genes. The AtNAC1 gene is predominantly expressed in the root tip and lateral root initials and a lower level of AtNAC1 transcripts has also been found in leaves and stems. Plants ectopically expressing AtNAC1 were more vigorous with larger leaves, thicker stems, and an increased number of lateral roots as compared to wild type (Xie et al., 2000; Figure 1A and C). Knock-down resulted in reduced lateral root formation (Xie et al., 2000; Figure 1A). The auxin-induced genes DBP (DNA-BINDING PROTEIN) and AIR3 (AUXIN-INDUCED IN ROOT CULTURES 3 ), encoding a DNA-binding protein and a subtilisin-like protease respectively, are AtNAC1 downstream targets (Xie et al., 2000). The AtNAC1 transcript and protein level is tightly controlled in time and space (Figure 1B). Transcriptional control involves auxin-mediated repressor degradation (Xie et al., 2000) leading to AtNAC1 proteins that form homodimers which induce transcription of the AtNAC1 gene itself and of downstream target genes. A dual survey system controls the protein level ensuring rapid signal suppression after auxin induction. On the RNA level, AtNAC1 mRNA was shown to be subjected to miR164-cleavage (Mallory et al., 2004; Guo et al., 2005). In the nucleus, through SINAT5 E3 ligase activity, AtNAC1 is specifically targeted for proteasomal degradation, thereby attenuating the AtNAC1 downstream responses (Xie et al., 2002) (Figure 1B). The importance of fine-tuning AtNAC1 signaling is demonstrated by transgenic plants in which the miR164 and AtSINAT5 levels were modulated (Xie et al.,

FIGURE 1: AtNAC1 ectopic expression (NAC1) and knock-down (Anti-3’) root phenotypes (A) compared to control (Ler and Vector left) A. thaliana plants after 14 days of growth. B, AtNAC1 regulation signaling cascade: auxin induces the SCFTIR1-targeted ubiquitination of the AUX/IAA repressors, and induces AtNAC1 transcription due to the auxin-responsive elements (ARE) in the promoter. The NAC1 mRNA level is controlled by miR164-cleavage activity. After translation, the NAC1 protein (blue box) transcriptionally activates its downstream targets, such as DBP and AIR3, resulting in lateral root formation. The protein level is controlled by the AtSINAT5 E3 ligase dimers, which target NAC1 for ubiquitination and proteasomal degradation. The auxin-induced NAC1 signaling is attenuated at mRNA level and at protein level (green lines). C, A. thaliana plants ectopically expressing NAC1 (NAC-O) are bigger with larger leaves compared to controls (vector) after 35 days of growth in soil. Scale bars are 0.5 cm. Images in A and C are reprinted from Xie et al. (2000).
2002; Guo et al., 2005). Ectopic expression of miR164 and AtSINAT5 resulted in a reduced number of lateral roots, and transgenic plants ectopically expressing a dominant negative mutant of AtSINAT5 and the miR164a and miR164b mutants contained more AtNAC1 mRNA and produced more lateral roots (Xie et al., 2002; Guo et al., 2005).

Legume plants do not only form lateral roots as secondary root organs, they also develop nodules as a result of a symbiotic interaction with rhizobia. Successful perception of the bacterial signal molecules, the Nod factors (NFs), by specific receptors in the epidermal root cells results in entry of the bacteria via the root hair curling process (Patriarca et al., 2004; Gage, 2004). Infection threads (ITs) guide the bacteria towards deeper cortical cell layers wherein a nodule primordium has been formed. Functional nodules develop when the ITs reach the nodule primordium and bacteria are taken up by the primordium cells and differentiate into bacteroids, which fix atmospheric nitrogen. Thus, two interlinked processes, rhizobial infection and organ formation, result in nitrogen-fixing nodules. The model legume Medicago truncatula develops indeterminate nodules that consist of different zones, a persistent apical meristem, an infection zone characterized by IT growth and where bacterial uptake takes place, the fixation zone in which nitrogen fixation is happening and a senescence zone, where bacteria and plant cells are degraded (Vasse et al., 1990; Gage, 2004).

Legume plants have recruited several pathways of other developmental processes to function in nodule formation. For instance, the rhizobial infection is derived from the ancestral arbuscular mycorrhizal interaction as mutants are available that are defective in both symbioses (Guinel and Geil, 2002). Moreover, the nodule organogenesis program might contain signaling pathways derived from stem organs, carbon storage sinks, or lateral root structures (Nutman, 1948; Sprent, 1989; Caetano-Anollés et al., 1993; Hirsch and LaRue, 1997). Lateral root and nodule formation show several similarities, such as their post-embryonic formation opposite the protoxylem poles through re-activation of the cell cycle in pericycle and/or cortical cells, respectively (Timmers et al., 1999; Dubrovsky et al., 2000). Furthermore, the number of both organs is regulated via a similar autoregulation mechanism (Nishimura et al., 2002; Penmetsa et al., 2003) and the initiation of both organs requires a local auxin accumulation (Mathesius et al., 1998; 2000; 2003; Kondorosi et al., 2005). The existence of common signaling mechanisms was further supported by mutant analyses, for instance the root inducer (RIND) Rhizobium etli mutant induced root outgrowths instead of nodules on Phaseolus vulgaris (Ferraioli et al., 2004) and the M. truncatula LATD mutant provided genetic evidence for the evolvement of a nodule meristem from a lateral root
meristem (Bright et al. 2005). Moreover, NFs were shown to stimulate lateral root formation through the same signaling pathway as used for the initiation of nodules (Olàh et al., 2005), and a range of genes, such as the MtLAX (de Billy et al., 2001), MtANN1 (De Carvalho-Niebel et al., 2002), MsCycA2;2 (Roudier et al., 2003) and ENOD11, ENOD12 and ENOD40, were induced in both developing organs (Journet et al., 1994; Papadopoulou et al., 1996; Hirsch et al., 2001).

In this perspective, we studied the involvement of NAC1-like transcription factors in the nodulation process. In silico analysis on M. truncatula databases revealed one Tentative Consensus (TC) sequence encoding an AtNAC1 homologue which was overrepresented in libraries derived from nodulated tissues. Here we present a detailed expression analysis of the MtNAC1 gene. Functional analysis, using ectopic expression and knock-down strategies, showed that MtNAC1 might be an important transcription factor in nodule development and rhizobial invasion.

**RESULTS**

**Sequence identification and molecular characterization of MtNAC1**

TBlastX analysis with the AtNAC1 coding sequence against the M. truncatula Gene Index database (MtGI; www.tigr.org) resulted in 16 TC clones, the deduced amino acid sequence of which showed similarity to AtNAC1. Only one clone, TC95634, showed similarity to AtNAC1 outside the NAC domain. We designated the clone MtNAC1. In silico expression of the TC clone showed that the clone frequently appeared in cDNA libraries from S. meliloti inoculated tissue (Figure 2A).

![FIGURE 2: MtNAC1 gene identification. A. In silico expression of MtNAC1 TC95634 showing the cDNA library name, the number of ESTs sequenced and the expression in percentage of the total library. MtBB, roots 4 days after inoculation with S. meliloti 1021; KV2, roots 2dpi with S. meliloti 1021; BNIR, nematode-infected roots; MTUS, M.truncatula Unigene set. B. Genomic structure of MtNAC1 showing the promoter region of 1500bps, the open reading frame (ORF) of 920bps, the position of putative AtmR164 complementarity in the mRNA, the primers used for amplification of the ORF (red) and the gene-specific 3’ probe used for RT-PCR and in situ hybridization and for the RNAi construct (green), and in blue the primers used for Q-PCR analysis of MtNAC1.](image-url)
The MtNAC1 open reading frame (ORF) has been isolated from M. truncatula cDNA (see Methods) and encodes a 306 amino acid protein with 73% similarity to AtNAC1. The deduced amino acid sequence contains the N-terminal NAC domain and a possible NLS for nucleus localization as predicted by PSORT (Figure 3).

**MtNAC1 expression in M. truncatula**

To obtain a first insight into the expression pattern of MtNAC1, quantitative RT-PCR was performed on cDNA derived from several M. truncatula Jemalong tissues (see Methods). Figure 4A shows expression in root tips, shoot apical meristems (SAMs), 1st leaves, and 2nd leaves.

**FIGURE 3:** Sequence alignment of NAC domain proteins showing the conserved N-terminal NAC domain sequence, which constitutes the black and grey boxed amino acids, showing 60% identity and similarity among the aligned sequences, resp. The amino acid sequence labeled with 'N' represents the NLS sequence motif. The proteins aligned include the Arabidopsis NAC1 (Xie et al., 2000), the M. truncatula MtNAC1 (this study), MtNAC2 (this study), and MtNAC3 (Genbank accession number AF254124; this study), the Petunia NAM (Soubrier et al., 1996), the ACCUC1 (Aida et al., 1997) and the Phaseolus vulgaris NAC1 (accession number AAK84863).
cotyledons of in vitro grown 5 day old M. truncatula plants, and in stems, leaves and flowers of 1 month old M. truncatula plants (see Methods). Higher expression levels were seen in root tips, leaves and cotyledons whereas a very low amount was found in mRNA derived from stems, SAMs, flowers and first leaves.

To investigate the involvement of MtNAC1 in nodule formation, the expression was analyzed in nodulated roots at various time points after inoculation with S. meliloti 1021 pHC60(GFP) (Cheng and Walker, 1998). Zone I regions of the root, i.e. the susceptible zone for nodulation with emerging root hairs, were harvested before inoculation, at the stage of rhizobial attachment (2dpi), at two consecutive stages of infection thread growth and primordium development (8dpi and 12dpi), and at the young nodule developing stage (16dpi). Moreover, the expression level was analyzed in young fixing nodules (22dpi) and in mature elongated nodules (28dpi).

MtENOD40 was used as a control for nodulation and showed an 8 fold upregulation at 8dpi, which increased to 36 fold at 12 dpi and decreased further at later nodulation stages (data not shown).

The MtNAC1 transcript level was about 2 fold higher in the 8 dpi stage compared to the level observed in uninoculated nitrogen-starved roots and a maximum of a 4 fold increase was seen at 12 dpi in developing nodules and in 16 dpi nodules after which the expression level
decreased but was still 2 times higher in 28 dpi nodules compared to control roots (Figure 4B).

As MtNAC1 is a potential ortholog of AtNAC1, we also controlled whether the gene is upregulated during lateral root formation and upon auxin application (see Methods). However, no upregulation of transcript level could be seen after 48h of 2 µM auxin application (Figure 4C) neither during lateral root formation (data not shown). In contrast, MtNAC1 was repressed 4h after auxin application to the roots (Figure 4C).

These experiments show that MtNAC1 is upregulated during early nodulation, in the stage of rhizobial infection and nodule primordium development, and that a high transcript level is retained in functional nodules. The expression is not restricted to nodules as transcripts were also detected in root tips, mature leaves and in a lesser extent in cotyledons. However, unlike AtNAC1, upregulation of transcript level could be detected neither upon auxin treatment nor during lateral root formation.

In situ localization and promoter-activity of MtNAC1

Nodule formation consists of two main pathways, rhizobial infection and primordium formation which merge when the bacteria are taken up by the plant cell and a young functional nodule is formed (Gage, 2004). To visualize where and when during nodule development MtNAC1 is expressed, in situ hybridization was performed to localize the MtNAC1 transcripts and the MtNAC1 promoter activity was analyzed. The promoter was isolated by a genome walking strategy (see Methods) and fused to the gus reporter gene by recombination in the Gateway pKm41GWS7 binary vector (Invitrogen, Karimi et al., 2002) which was used for A. rhizogenes transformation of M. truncatula roots (see Methods).

A high level of MtNAC1 promoter-gus activity was observed in the vasculature of the main root (Figure 5A). This expression pattern was not confirmed by in situ hybridization (Figure 5B and C) and probably corresponds to a background expression often observed when promoter-GUS constructs are analyzed in transgenic roots. No blue staining was observed in the lateral root primordium nor in emerging lateral roots (Figure 5A and D). Also by in situ hybridization no transcripts could be detected in those tissues (data not shown). In young nodules GUS staining was seen in a small zone in the apical part of the nodule (Figure 5E and F). In situ hybridization on longitudinal sections of mature nodules showed transcripts in the infection zone and in early fixation zone (Figure 5G, H, I and J).

Thus MtNAC1 gene expression was absent in the root tissue and in lateral root primordia, but was found back in the infection zone of the nodule. This zone is characterized
by cells which contain infection threads and where bacteria are taken up by the plant cells and develop into bacteroids.

FIGURE 5: MtNAC1 transcript localization and promoter activity in M. truncatula roots and nodules. Stereomicroscopical pictures in A, D, E and F show the MtNAC1 promoter activity which is indicated by gus-staining of transgenic M. truncatula roots and nodules. Image B, C, and G to J show bright- and darkfield microscopy images of in situ hybridisation on 10 µm sections of M. truncatula root and nodule tissue with the MtNAC1 antisense RNA probe. A, blue staining in the vascular tissue of the main root; B and C, uninoculated roots without signal for MtNAC1 transcripts. D, promoter-activity is absent in the lateral root primordium. E and F, 22 dpi nodule showing gus-expression in the vascular tissue of the transgenic M. truncatula root and in the apical part of the nodule tissue. G, H, I and J, images of young nodules showing signal (white dots, arrows) in the infection zone (i). The observation of gus-staining in the vascular tissue of the main root was not confirmed by the in situ hybridisation experiment and presumably is an artifact of the promoter-gus method, because often the promoter-gus analysis of different genes in hairy roots resulted in this background expression. m, meristem; f, fixation zone; vb, vascular bundle.
Ectopic expression and RNAi of MtNac1 in transgenic M. truncatula roots

To obtain an insight into the function of MtNAC1 during nodulation, MtNAC1 expression was modulated by introducing an overexpression or RNAi construct in transgenic roots and by analyzing nodule formation on those roots (see Methods). To obtain ectopic expression, the MtNAC1 ORF was introduced in the Gateway pB7WG2D binary vector (Invitrogen, www.psb.ugent.be/gateway/) in which the ORF expression is driven by the 35S constitutive promoter. The plasmid also contains an egfp marker gene on the T-DNA region.

For knock-down/out of MtNAC1, an RNAi construct was made by site-specific recombination of a fragment containing the 3’region of MtNAC1 in the Gateway pK7GWIWG27F2 vector (Karimi et al., 2002; see Methods), which also contains an egfp marker gene. The binary vectors were introduced in the A. rhizogenes A4TC24 strain (see Methods). Transgenic root lines were screened for GFP expression by stereomicroscopy using UV light emission and were inoculated with S. meliloti 1021 one month after the A. rhizogenes transformation (see Methods). In the population of the control roots, 20 days after inoculation with S. meliloti, 17 out of 20 lines (85 % of the lines) showed an average of 4 ± 0.69 healthy fixing nodules, as seen by the pink colour of the fixation zone which is indicative for the presence of leghemoglobin (Figure 6A and B), and 3 lines (15 % of the lines) showed an average of 4 non-fixing nodules, probably as a consequence of the suboptimal conditions for nodulation on transgenic roots. Only 3 out of 16 MtNAC1 ectopic expressing lines (19 %) contained only one healthy fixing nodule per plant (Figure 6A). Three out of 16 lines (19 %) showed no nodule formation at all, 5 lines (31 %) developed on average 3.2 ± 0.73 non-fixing (fix-) nodules and 5 lines (31 %) arrested at nodule primordium stage (on average 2 ± 1 NP per line; Figure 6A, B and C). qRT-PCR analysis of the ectopic expression lines and control lines revealed indeed higher levels of MtNAC1 in the transgenic lines compared to the control lines (Figure 6D). Within the transgenic lines, there were differences in the MtNAC1 transcript level, but there was no clear correlation between the level of overexpression and the nodule phenotype (i.e. no nodules, nodule primordia or fix- nodules). Sectioning showed that the arrest at the primordium stage corresponded to uninfected cells through which infection threads were passing (Figure 6B and C compared to E and F). The nodule primordium was arrested and did not develop into a central tissue containing a nodule meristem (Figure 6B compared to E). The infection threads had a normal appearance compared to the ITs observed in control nodules (Figure 6C compared to F).
The MtNAC1 knock-down/out effect on nodulation was analyzed in 47 transgenic root lines and compared to the nodulation capacity of 33 control (pK7GW2D-GUS) lines, containing an egfp marker gene and the gus gene, at 26 days after inoculation with S. meliloti 1021 (see Methods). 82% of the control roots showed an average of 3.58 ± 0.41 healthy functional nodules per plant, whereas 18% of the lines showed non-fixing nodules (Figure 7A). The healthy nodules were elongated and contained a fixation zone (Figure 7B). For the RNAi lines, different phenotypes were observed. 17% of the RNAi lines showed wild type nodulation, 34% of the lines showed no nodule formation, 34% of the lines only developed white non-functional nodules (Figure 7C). The average amount of non-fixing nodules that formed on this fraction of the population was equal to the number of nodules formed on control lines (3.81 ± 0.68). Finally, 7 lines or 15% formed ectopic roots that developed from nodule primordia (Figure 7D and E). Bright-field microscopy on sections of these ectopic roots showed a lot of new primordia (Figure 7F, G and H) and few infection threads in the epidermal and outermost cortical cells (Figure 7I, arrowheads).
qRT-PCR was performed on 23 lines to analyze the MtNAC1 transcript levels in the RNAi lines and transcript levels were compared to the levels observed in control roots. Next, the expression levels were correlated to the nodulation phenotype (Figure 7J). In 4 lines (N4, NK1, NK7 and NK10), no reduction in MtNAC1 mRNA was observed, and these lines developed wild type nodules. Wild type nodules were also observed in the 4 lines with a reduced mRNA level to a maximum of 65% of normal expression (N5, NN13, N1 and N9). A reduction of the MtNAC1 mRNA level to 30%-65% was reached in 11 lines, displaying a mixture of phenotypes, including no nodule formation, non-functional white nodule formation or the formation of ectopic roots from the nodule primordia. When a reduction of more than 70% of MtNAC1 was reached no nodule formation was observed (NN14, N6, NN6 and N3).

In conclusion, functional nodules were only formed when at least 70% of the expression level of MtNAC1 was retained. Lower expression levels resulted in non-fixing nodule formation or root initiation after nodule primordium formation or in a complete nod phenotype.

**DISCUSSION & CONCLUSIONS**

Plant development is tightly regulated by specific sets of transcription factors (TFs). One of the largest families are the NAC transcription factors, which play roles in SAM formation, organ boundary specification, and lateral root development, but also in responses to the environment (Souer et al., 1996; Aida et al., 1997; Olsen et al., 2005). Legumes, such as M. truncatula, might have recruited gene functions or signaling mechanisms from pre-existing developmental pathways for nodulation. A nodule resembles a lateral root in several aspects, such as in similar positioning on the root, in auxin-dependent organ initiation, in primordium formation, and common autoregulatory signaling pathways for development, aspects that were already extensively discussed in literature, and for which evidence on the genetic level is accumulating (Journet et al., 1994; Mathiesius et al., 1998; de Billy et al., 2001; Kondorosi et al., 2005). Here we show that MtNAC1, a homologue of AtNAC1 which functions in lateral root development, has an important function during the development of nodules.
FIGURE 7: MiNAC1 RNAi nodule phenotype on transgenic M. truncatula roots.

A. The percentage of transgenic lines for each nodule phenotype observed in control (green) and MiNAC1 RNAi (red) transgenic roots. For the population of control lines, 82% developed on average 3.6 healthy fixing nodules, but 18% of the lines developed non-fixing nodules. For the RNAi population, only 17% showed wild type nodulation, whereas 34% did not develop nodules, 34% showed non-fixing nodules (*, on average 3.8 nodules, which is equal to the numbers on control roots), and 15% developed ectopic roots from the primordia.

B to I, stereomicroscopical images of 26 dpi nodules on control roots (B), RNAi NN7 (C), which had non-fixing nodules, on NN3 (D) and NN2 (E), both showing ectopic roots. Image C shows non-functional white nodules formed on roots of NN7 with a 69% reduction level. Images D and E show the ectopic roots that appear on the previously initiated nodule primordia on the roots with a reduced expression level of 65%. F to I demonstrate bright-field microscopy on 5 µm sections of ectopic roots. F, new roots developed from the primordia. G, NN10 ectopic root structure showing a lot of new primordia (arrows). H, ectopic root structure of the NN3 RNAi line, showing new roots that arise from the primordia. I, bacteria (arrowheads) are trying to invade the epidermal tissue.

J, qRT-PCR analysis for MiNAC1 expression on control transgenic roots (c10, c1 and c7) and on 23 different RNAi lines (named N, NN or NK). Relative expression values compared to a control level value of 1 are shown. Error bars, standard deviations on technical triplicates.
MtNAC1 is not the orthologue of AtNAC1

M. truncatula AtNAC1 homologues were searched through TBlastX analysis using the AtNAC1 sequence on M. truncatula EST databases (www.tigr.org; Journet et al., 2002). The ORF of one EST encodes a 306 aa protein with 73 % similarity to AtNAC1. The encoded protein contains the conserved NAC domain and a predicted NLS motif, indicating that it might also function as a TF in M. truncatula. However, MtNAC1 is probably not the orthologue of AtNAC1. qRT-PCR on different M. truncatula tissues revealed MtNAC1 transcripts in leaves and root tips, as observed for AtNAC1 (Xie et al., 2000). However, neither transcripts, as observed by in situ hybridization, nor promoter-activity was detected during lateral root differentiation, a process during which AtNAC1 has an important function (Xie et al., 2000). Furthermore, in contrast to AtNAC1, the MtNAC1 expression level did neither raise in auxin-treated roots nor during auxin-induced lateral root formation. Also the functional analysis demonstrated that MtNAC1 might have a completely other role than AtNAC1 as modulating the MtNAC1 transcript level did not result in a lateral root phenotype. All together, these data show that MtNAC1 is not the orthologue of AtNAC1.

MtNAC1 expression suggests a nodulation-related function

Interestingly, qRT-PCR on S. meliloti inoculated tissue demonstrated a 4 fold increase in transcript level during early nodule development as compared to uninoculated roots. Between 2 and 8 days after inoculation, the MtNAC1 expression level was elevating and peaked around 12dpi, the stage at which infection threads spread between the nodule primordia cells. Also in mature nodules the MtNAC1 expression level was still higher compared to the level in roots. In situ localization and promoter-gus analysis showed that the gene is active in cells of the infection zone and early fixation zone. In the near future, transgenic roots containing the promoter-gus fusion will be inoculated with an S. meliloti strain carrying the dsred (encoding the Discosoma sp. red fluorescent protein) marker gene, to analyze the MtNAC1 expression during the rhizobial infection and invasion of the nodule primordium by fluorescence microscopy.

Taken together, a nodulation-related expression pattern was discovered for MtNAC1 as the gene is upregulated at the onset of symbiosis and is expressed in the infection zone of the mature nodule, thereby suggesting a recruitment of this function for nodule development.
MtNAC1 ectopic expression and knock-down revealed an essential role during the nodulation process

A. rhizogenes transformation of M. truncatula results in the formation of chimeric plants consisting of a wild type shoot and transgenic roots. This is a fast and easy method to modulate gene expression in the root and to examine the effect on root and nodule development (Boisson-Dernier et al., 2001). Transgenic roots ectopically expressing or downregulating MtNAC1 revealed different nodulation phenotypes.

Analysis was performed at 20 or 26 days after S. meliloti inoculation. Lines with ectopic expression of MtNAC1 resulted in 4 groups of phenotypes, 19 % of the lines had only one healthy fixing nodule, 31 % only developed nodule primordia, 31% of the lines developed fix- nodules, and 19 % of the lines did not nodulate at all. qRT-PCR analysis revealed that transcript levels were efficiently elevated, implying that 35S promoter is suitable for modulation of the transcript levels in M. truncatula roots.

The nod- phenotype as well as the arrested nodule primordia shows that initial steps of the nodulation pathway are affected when MtNAC1 is ectopically expressed. Sectioning through arrested primordia showed that a nodule primordium has been formed and that normally looking infection threads were penetrating the cortex but that no further development of the nodule took place.

In the MtNAC1 RNAi population, 17 % of the lines showed wild type nodulation, whereas 34 % of the lines developed white non-functional nodules, an additional 34 % had no nodules, and 15 % of the lines formed ectopic roots from initiated nodule primordia. qRT-PCR analysis revealed that a 35 % reduction of MtNAC1 transcription was sufficient to hinder functional nodule formation. In lines with less than 30 % transcripts compared to control lines, no nodules did develop. Also for the knock-down, the strongest phenotype was nod-.

The non-functional fix- nodules observed in some knock-down lines and in some lines ectopically expressing MtNAC1 were not characterized yet because this phenotype could be or the consequence of the modulation of the MtNAC1 transcript level or be caused by the suboptimal nodulation conditions of hairy roots as also 15% of the control lines developed fix- nodules. Stably transformed plants are awaited for further analysis.

Bright-field microscopy of the ectopic roots showed that several nodule primordia were initiated but switched to root development. A few infection threads were formed that aborted in the peripheral region of the root tissue, indicating that initial infection had happened but that further IT growth was arrested. A related observation has been made in
Phaseolus vulgaris, where the bacterial R. etli Root inducer (RIND) mutant induces the formation of abortive ITs and the formation of ectopic roots from the nodule primordia (Ferraioli et al., 2004). Also, upon addition of purified NFs, lateral root formation may be stimulated (Olàh et al., 2005), indicating that the initial trigger with NFs is not always sufficient for nodule development, and that a continuous signaling from the invading bacteria is necessary for nodule-specific differentiation.

Taken together, modifying MtNAC1 transcript levels resulted in a serious disruption of nodule development. The fact that both, ectopic expression and knock-down, displayed nod- phenotypes implements that MtNAC1 transcript levels are tightly regulated during M. truncatula nodulation. MtNAC1 transcripts were detected in the infection zone and early fixation zone, which is the tissue that is characterized by IT penetration and cellular differentiation. Yet it is possible that gene activation is directly dependent on the main bacterial signal molecules, the Nod factors. Therefore, it will be analyzed whether NF’s will be sufficient to induce MtNAC1, or whether other bacterial signals are required. Whether the nod- phenotype is the effect of a defective infection and/or primordium formation has not been established and will be analyzed in the stably transformed plants that will be obtained soon.

Nod factors (NFs) are perceived by epidermal root hair cells and set in motion local responses such as ENOD11 gene induction, root hair curling and IT formation and distant responses such as pre-infection thread formation and induction of cortical cell division (Schultze and Kondorosi, 1998; Charron et al., 2004; Gage, 2004; Oldroyd and Downie, 2004; Geurts et al., 2005). These distant responses are probably provoked by NF-induced gradients of secondary signals that prepare the cortical tissues for nodulation, for instance by changing the hormonal balances (Libbenga et al., 1973; Timmers et al., 1999). Also downstream from the epidermis, NF signaling from bacteria is important for proper nodule development. In S. rostrata, it has been shown that NF’s produced within the IT’s are locally needed for proper IT structure but also to synchronize infection and primordium development (J. Den Herder et al., submitted). Besides the NFs, also other signaling molecules from the bacteria, such as lipopolysaccharides (LPS), contribute to distant signaling (Leigh and Walker, 1994; Mathis et al., 2005).

Hence, a continuous communication between the rhizobial infection path and the developing organ has to be maintained to enable the differentiation of the infected primordium into a central nodule tissue where bacterial uptake and bacteroid differentiation can take place.
As MtNAC1 expression is connected to cells containing IT’s in the infection zone, the question rises whether MtNAC1 is locally needed for e.g. IT growth or whether it is involved in the distant signaling and communication between the infection and the nodule differentiation. Mutants that are defective in local responses are also nod^−, but often in weaker phenotypes ITs are observed that are irregular in shape (Tansengco et al., 2003; Kuppusamy et al., 2004; Veereshlingham et al., 2004). This phenotype has not been observed in MtNAC1 but further microscopy has to be performed. More plausible is that MtNAC1 is important for installing or maintaining hormone signaling gradients necessary for PIT and primordium formation and to keep the infection and the cell division in harmony. Such a hypothesis is inspired by the observation that in some MtNAC1 RNAi lines, the primordium changes into a developing root. Auxin and cytokinin play a pivotal role in initiation of the first cell division in the cortex (Ferguson and Mathesius, 2003). Auxin is also locally needed during the onset of rhizobial infection (Van Noorden et al., 2006) and in mature nodules auxin was localized in the infection zone (Fedorova et al., 2005). Cytokinin induces cortical cell division, influences nodulin gene expression (Bauer et al., 1996; Fang and Hirsch, 1998). Exogenous application of cytokinin increases nodule numbers (Lorteau et al., 2001).

The potential influence of MtNAC1 on the auxin and cytokinin landscape during nodule development will be examined by introducing the auxin- and cytokinin-responsive promoter-gus fusions, DR5-gus and ARR5-gus (Ulmasov et al., 1997; Lohar et al., 2004), respectively, in the transgenic MtNAC1 ectopic expressing and RNAi lines. Another approach involves exogenous application of cytokinin and/or auxin during nodulation of roots ectopically expressing or downregulating MtNAC1. Moreover, it will be interesting to know whether the MtNAC1 expression level is increased before the rise in e.g. MtENOD40 transcript level as this gene is a marker for the elicitation of NF-elicited distant responses.

Several epidermal putative NF receptors have been isolated, some of which might be involved in IT formation while others are involved in provoking distant responses (Amor et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). For instance, the HCL/LYK3 gene, encoding a LysM domain containing receptor-like kinase, is proposed to function as an entry receptor because the epidermal NF responses still occur in the HCL mutant, but IT formation and cortical cell division are impaired (Catoira et al., 2001; Limpens et al., 2003). Moreover, also downstream signaling components have been cloned, such as the DMI1, DMI2 and DMI3 genes which code for respectively a ligand-gated cation channel, a leucine-rich repeat receptor-like kinase (LRR-RLK) and a calcium-calmodulin-dependent protein kinase (Ané et
al. 2004, Endre et al. 2002; Lévy et al. 2004). Furthermore, two transcription factors important for nodulation belonging to the GRASS family have been characterized and are encoded by the NSP1 and NSP2 genes (Kaló et al. 2005; Smit et al. 2005). It will be interesting to analyze the expression of MtNAC1 in the corresponding M. truncatula mutants, and in spontaneous nodules that occur on the Medicago sativa subspecies varia A2 line (Kevei et al., 2002), and in dominant dmi3 mutants that show constitutive kinase activity (meeting report 3rd International Conference on Legume Genetics and Genomics, Brisbane 2006). Also the expression in those nodules has to be analyzed.

All together we show that MtNAC1 might be an important TF in the nodule formation process. To determine what are its downstream targets, affymetrix experiments comparing transcript profiles of nodulated transgenic plants with a knock-down/out of MtNAC1 and nodulated wild type M. truncatula should be performed. Likewise sets of genes that are involved in the control of signaling gradients, which are required for the development and functioning of a nodule, will be discovered.
ADDENDUM: Molecular characterization and functional analysis of two additional NAC genes in *M. truncatula*

RESULTS

Identification and molecular characterization of MtNAC2 and MtNAC3

The TBlastX analysis on the MTGI database with the AtNAC1 gene revealed 16 NAC gene sequences. The first hit was MtNAC1 and has been described in the first part of this chapter. The second hit, TC95256, designated MtNAC2 contains an ORF of 1152 bps which encodes a NAC transcription factor of 383 aa with 53 % similarity to AtNAC1, 46 % to AtNAC2, and 50 % to MtNAC1 (Figure 3). The in silico expression did not show higher transcript levels in nodulation libraries. Some MtNAC2 clones were present in a cDNA library of developing root tissue (0.21%) (Figure 8A).

Another TC sequence, TC10777, was annotated as a MtNAC1 orthologue by the TIGR association (www.tigr.org). This TC was not found after TBlastX analysis with the AtNAC1 sequence. The clone, designated MtNAC3, contains an ORF of 1334bp encoding a 444 aa protein with 43 % similarity to AtNAC1, 44 % to AtNAC2, 40 % similarity to MtNAC1, and 30 % similarity to MtNAC2 (Figure 3). The similarity with these other NAC proteins was mainly observed in the conserved NAC domain. The C-terminal end was longer compared to other NAC1-proteins and showed less similarity. ESTs were found in cDNA libraries of 3dpi (KV3) and 1dpi (KV1) nodulated tissue (0.03 and 0.07%, resp.) (Figure 9B), and therefore the TC was selected for further study.

The genomic sequence of MtNAC2 was available from the AC145753.1 clone (Genbank; www.ncbi.nlm.nih.gov) and the genomic MtNAC3 sequence was isolated by PCR on M.
Medicago truncatula Jemalong J5 genomic DNA. Both genes contain two introns at the conserved positions defined for NAC family members (Duval et al., 2002) (data not shown).

To define whether these are single-copy genes, Southern analysis with a probe complementary to the 3’ region was performed (see Methods, Table 1). Hybridisation with the MtNAC2 probe revealed three bands (one strong and 2 weak) for the EcoRV and HindIII digested genomic DNA and one strong band for the HindII digested genomic DNA. For MtNAC3, the hybridization signal detected on HindI and EcoRV digested genomic DNA was weak, and located at 2200bp and 9000bp, respectively, and the HindIII digested genomic DNA showed 1 strong and 1 weak hybridization signal. Hence, a small gene family of both MtNAC genes is most likely present in M. truncatula (Figure 9).

**MtNAC2 and MtNAC3 expression in M. truncatula**

To analyze the expression of both clones during nodule formation, RT-PCR has been performed on nitrogen-starved roots and on roots inoculated with S. meliloti 1021 1, 2, 3, 4 and 14 days post inoculation (Figure 10A and B) and in a second experiment Q-PCR analysis was done on cDNA of zone I regions of the root, i.e. the susceptible zone for nodulation with emerging root hairs, before inoculation, at the stage of rhizobial attachment (2dpi), at two stages of infection thread growth and primordium development (8dpi and 12dpi), and at the young nodule developing stage (16dpi). Moreover, the expression level was analyzed in young fixing nodules (22dpi) and in mature elongated nodules (28dpi) (Figure 10C and D).

As shown in Figure 10A, the level of MtNAC2 at 1dpi is already higher compared to the level observed in uninoculated roots. At 12dpi, an even higher induction level of MtNAC2 transcripts was observed and the transcript level stayed high throughout the nodule developmental process (Figure 10A and C). The MtNAC3 transcript level raised slightly later to a maximum level of 2-4 times the level in uninoculated roots. This level was kept during the complete nodulation process (Figure 10B and D).
These results indicate a nodulation-related expression for both NAC genes. Therefore, in situ hybridization on *M. truncatula* uninoculated and nodulated root tissue was performed with specific RNA antisense probes (see Methods). The signal for *MtNAC2* transcripts in uninoculated roots was found in the vascular bundle of the main root (Figure 11A, B, C and F). Transversal sectioning (Figure 11C and F) showed that the signal is located around the meta- and protoxylem vessels, but also at the phloem and protophloem cells. No signal has been detected in the lateral root (Figure 11A), neither in developing lateral root primordia (data not shown). The absence of *MtNAC2* expression in lateral root formation was also observed by qRT-PCR on a series of samples of an auxin-dependent lateral root induction assay (data not shown).

In the young nodule no transcripts were observed in the nodule itself, but the vascular tissue connecting the root and nodule vasculature showed a weak signal (Figure 11D and E).

For *MtNAC3*, a patchy expression was observed along the vascular bundle of the main root in uninoculated roots (Figure 11G and H). Transversal sections revealed that this signal is located in the phloem and protophloem cells and around xylem tissue at different stages of differentiation (protoxylem, metaxylem, proto- and metaxylem vessels) (Figure 11I and L). At places where young nodules arose, the signal in the vascular tissue of the main root became more pronounced at the nodulation site (Figure 11J and K).
In conclusion, MtNAC2 and MtNAC3 transcripts are associated with the root and nodule vasculature, more specifically, in the phloem and protoxylem cells and also around differentiating and differentiated xylem tissue. For MtNAC2, the signal was detected along the root length, whereas MtNAC3 transcripts were observed in a patchy pattern over the root length, a signal that was more intense at the nodulation sites.

**FIGURE 11:** Bright- and darkfield microscopy images of in situ hybridisation on 10 µm sections of M. truncatula root and nodule tissue with the MtNAC2 (A to F) and MtNAC3 (G to L) antisense RNA probes. A, B, C and F show signal (white dots) in the vascular bundle (vb) of the main root (arrows), but not in the lateral root (LR). Image C and F show a transversal section through the vascular bundle of the main root from which a lateral root is emerging. Signal for MtNAC2 transcripts is mainly seen around the meta- (mvx) and protoxylem vessels (pxv), but also in the phloem (p) and the protoxyloem cells (pp). Image D and E show the signal in the vascular bundle (arrows) connecting the 14dpi nodule and the root vasculature. Longitudinal (G and H) and transversal (I and L) sections of uninoculated roots show a patchy signal (arrows) for MtNAC3 transcripts in the vascular bundle (vb) of the main root. In L it is shown that as for MtNAC2 the signal is located in the phloem and protoxyloem, as well as around the different forms of differentiating xylem tissue. Image J and K show a strong upregulation of MtNAC3 in the vascular bundle (vb) (arrows) at the 14dpi nodule site in the main root. C, root cortex.
Nodulation of transgenic *M. truncatula* roots ectopically expressing or downregulating MtNAC levels

The ORF of MtNAC2 and MtNAC3 was cloned in the Gateway pB7WG2D binary vector wherein the ORF is driven by the constitutive 35S promoter (see Methods). The constructs were introduced in *M. truncatula* hairy roots and the effect of ectopic expression was studied upon inoculation (see Methods). RNAi constructs were obtained by cloning the gene-specific 3′ region (Table 1) in the Gateway pK7GW1WG27F2 binary vector and were introduced in *M. truncatula* hairy roots. Both vectors also contained an egfp marker gene allowing in vivo screening of cotransformed hairy roots.

Eleven transgenic root lines containing the MtNAC2 overexpression construct, 23 MtNAC2 RNAi lines, and 33 control lines transformed with the pK7GW2D-GUS vector, containing an egfp marker gene and the gus gene, were obtained and analyzed after inoculation with *S. meliloti* 1021. For 82% of the control population, an average of 4 normal pink colored (reflects the presence of leghemoglobin) fixing nodules was observed 25 days post inoculation, and 18% of the roots developed white non-fixing nodules, possibly a consequence of the reduced nodulation capacity of transgenic roots (Figure 12A). One MtNAC2 ectopic expressing line showed normal fixing nodules 25 days after inoculation, while 2 lines developed white nodules (Figure 13A) and the other 8 did not nodulate (73% of the lines; Figure 12A).

34% of the MtNAC2 RNAi lines did not develop nodules at 25dpi, 34% lines displayed white nodules, and 26% showed pink fixing nodules, resembling wild type nodules (Figure 12A). qRT-PCR analysis on 6 RNAi and on 3 control lines revealed that two RNAi lines showed an equal MtNAC2 mRNA level compared to the transcript level in control lines, indicating that the silencing mechanism was not active in these lines (Figure 13B). The other 4 MtNAC2 RNAi lines showed a reduction in the transcript level of 65% to 80% (Figure 13B). Three out of these 4 lines did not develop nodules, and one line only had one white nodule. Additionally, the transgenic roots with the most severe knock-down developed lots of lateral roots.

In conclusion, MtNAC2 ectopic expression and RNAi lines inoculated with *S. meliloti* resulted in an inhibition of nodule formation.

The influence of MtNAC3 ectopic expression and knockdown on the nodulation phenotype was analyzed in 9 transgenic root lines containing the overexpression construct and in 35 lines containing the RNAi construct, and was compared to 35 hairy root lines transformed with the control vector. At 25dpi, all the lines ectopically expressing MtNAC3
showed normal pink coloured fixing nodules (Figure 12B). In 4 of these lines the nodules appeared in a pairwise manner, with always one white small nodule next to a pink elongated nodule (Figure 13C).

![Figure 12](image1.png)

**FIGURE 12:** The percentage of transgenic lines for each nodule phenotype observed in control (grey), MtNAC2 ectopic expression (OE) lines (pink, A), MtNAC2 RNAi lines (white, A), MtNAC3 ectopic expression (OE) lines (pink, B), andMtNAC3 RNAi lines (white, B).

In the population of RNAi lines, only 19 % of the lines showed wild type nodulation, 31 % of the lines developed white non-fixing nodules (Figure 13D) and 43 % of the lines did not nodulate (Figure 12B). The MtNAC3 mRNA level was analyzed by qRT-PCR in 10 RNAi lines and 2 control lines (Figure 13E). Line 2 and 11 showed a 20% reduction and a normal mRNA level respectively, as compared to control levels, and nodulated normally. The lines with a moderate reduction in the MtNAC3 transcript level (60, 70 and 75% reduction in lines 1, 8 and 4 resp.; Figure 13E) showed both fixing and non-fixing nodules. The knock down line 3 and 9 only contained 16% and 18% of the MtNAC3 mRNA level compared to control levels, but these lines had 3 and 5 normal fixing nodules, respectively.

![Figure 13](image2.png)

**FIGURE 13:** Ectopic expression and RNAi of MtNAC2 and MtNAC3 in M. truncatula transgenic roots. **A,** stereomicroscopical image of 25dpi fix nodule formed on roots containing the MtNAC2 overexpression construct. **B,** relative expression levels of MtNAC2 in control and RNAI hairy root lines. **C** and **D,** stereomicroscopical images of 25dpi nodules on transgenic M. truncatula roots transformed with the MtNAC3 overexpression (C) or RNAI (D) construct. C shows a normal pink elongated functional nodule with a small white nodule positioned right next to it on the root. D shows a fix nodule on RNAI line 8, which displayed a moderate reduction of MtNAC3 mRNA level. **E,** relative expression levels of MtNAC3 in control and RNAI hairy root lines. Error bars, standard deviations on 3 technical replicates.
DISCUSSION AND CONCLUSIONS

Apart from MtNAC1, 2 other MtNAC genes, designated MtNAC2 and MtNAC3, were withdrawn for analysis. Within the TIGR database, cDNA clones encoding MtNAC2 were frequently found in cDNA libraries of developing roots, whereas MtNAC3 cDNA clones were present in nodulation-related cDNA libraries. The corresponding genomic sequences enclosed 2 introns, located in the coding sequence at conserved positions characteristic for NAC-domain encoding genes (Duval et al., 2002). Southern hybridization revealed a small gene family for these NAC genes and both deduced amino acid sequences showed a low degree of similarity to the A. thaliana AtNAC1 and AtNAC2 amino acid sequences. Moreover, the similarity between MtNAC2 and MtNAC3 was low, even so the similarity of both amino acid sequences to MtNAC1.

**MtNAC2 and MtNAC3 exhibit a nodulation-related expression**

qRT-PCR analysis showed that MtNAC2 was upregulated very early after inoculation and an elevated transcript level was detected throughout the nodulation. However, in situ hybridization only showed a very weak signal in the root vasculature and no signal in the nodule. Moreover, the signal in the vasculature was difficult to visualize as a lot of reflection occurred due to the cell wall thickening of xylem cells. The MtNAC3 transcript level raised slightly later than the MtNAC2 level upon S. meliloti inoculation, and remained approximately at a 2-3 fold higher level throughout nodulation compared to the level observed in uninoculated roots. By in situ hybridization, MtNAC3 transcripts were also localized in the vascular tissue of the main root, and high signal was detected in the vasculature of the root at the sites where mature nodules were present. In contrast to MtNAC2, MtNAC3 transcripts were found back in a patchy pattern along the vasculature of the main root. Thus, both genes were primarily expressed in the vascular tissue of the root and in the nodule connecting vascular tissue.

In situ hybridization was not sufficiently sensitive to precisely locate the gene-specific transcripts inside the vascular bundle. MtNAC2 signal was very weak, making it difficult to distinguish from background reflection. It was also difficult to predict in which cells of the vasculature both genes were expressed because of the low morphological preservation of slides that are subjected to in situ hybridization. Promoter-gus fusion analysis has to be performed to be able to predict a more precise localization of the gene expression.
Nodulation-specific functioning of MtNAC2 and/orMtNAC3 in S. meliloti-inoculated transgenic M. truncatula roots

Ectopic expression and RNAi of both MtNAC genes in transgenic M. truncatula roots revealed different nodulation phenotypes. MtNAC2 ectopic expression resulted in a nod\(^{-}\) phenotype in 73% of the lines and knock-down of MtNAC2 caused nod\(^{-}\) roots in 34% of the lines and fix\(^{-}\) nodules in 34% of the RNAi hairy root lines. qRT-PCR on the RNAi lines suggested that 65% reduction of the mRNA level was sufficient to block nodulation. Therefore, the nod\(^{-}\) and fix\(^{-}\) phenotypes observed suggest a potential nodule-specific function for MtNAC2. As only 11 lines were analyzed for the ectopic expression, still more lines should be analyzed to be able to draw a conclusion. Moreover, a detailed analysis has to be performed to try to unravel at which level in the nodulation pathway the plants are blocked. As it is difficult to analyze the early steps of the invasion in hairy roots, stably transformed plants have to be created. These plants will also allow to analyze lateral root formation, which might be influenced in the RNAi lines, but which could not be analyzed yet because of the natural heavy branching of hairy roots.

MtNAC3 ectopic expression on the other hand did not alter the nodulation capacity of the transgenic roots, and introducing an MtNAC3 RNAi construct resulted in 43% nod\(^{-}\) lines and 31% fix\(^{-}\) lines. However, qRT-PCR on these lines revealed that functional nodules could still be formed when a reduction to 16% of the normal level was achieved in the roots. Hence, we concluded that overproduction of MtNAC3 transcripts could not block or inhibit the nodulation process and knock-down showed nod\(^{-}\) and fix\(^{-}\) nodule phenotypes, but also normally functioning nodules. This suggests that MtNAC3 does not directly affect nodulation or is functionally redundant.

In conclusion, further analysis of promoter-gus fusions and of stably transformed plants with a modulated expression level of the NAC genes will provide a better insight in their putative nodulation function. Based on our current results and on the literature, we might just speculate on a function in signaling necessary for communication between the nodule and root or other organs, such as the shoot, and which is possibly related to metabolite status sensing. Communication between the vascular tissue of the root and nodule is required for development of the fixation zone, in which the bacteria produce and require specific metabolites, and is also necessary during nitrogen fixation in the infected cells, where the vascular phloem is involved in sensing of the carbon/nitrogen metabolic status of the plant (Streeter, 1993). One example of a TF known to be involved in this
process concerns the vascular bundle-associated *M. truncatula* Krüppel-like zinc finger TF, which is required for the formation of the central nitrogen-fixing zone of the root nodule and also shows a fix− phenotype upon antisense (Frugier et al., 2000).

**METHODS**

**Plant material, bacterial strains, and growth conditions**

*S. meliloti* 1021 and *S. meliloti* 1021 (pHC60-GFP) (Cheng and Walker, 1998) were grown at 28° C in YEB (Vervliet et al. 1975). *Medicago truncatula* Jemalong J5 growth and inoculation were performed as described (Mergaert et al., 2003). Root tips, shoot apical meristems, first leaves and cotyledons were harvested from in vitro grown 5 day old *M. truncatula* J5 plants, and stems, leaves and flowers were sampled from 1 month old *M. truncatula* J5 plants grown in nitrogen-rich soils. In vitro growth was done in square petri dishes (12 × 12 cm) on Kalys agar (HP 696-7470 Kalys, France) containing SOLi medium (as described at www.isv.cnrs-gif.fr/embo2/manuels/index.html) supplemented with 1 mM NH₄NO₃ at 25° C in a 16h photoperiod and a light intensity of 70 μE per s m⁻². Whole roots, zone I root regions, or nodules were harvested from 30 plants per stage. Screening for the presence of GFP bacteria was performed with a stereo microscope MZFLII (Leica, Wetzler, Germany) equipped with a blue-light source and a Leica GFP Plus filter set (λ_ex =480/40; λ_em= 510nm LP barrier filter). For auxin-induction series, in vitro grown 3-day-old *M. truncatula* J5 plants were transferred to new plates containing 2 µM α-naphtalene acetic acid (NAA, DUCHEFA Biochemie, Haarlem, NL) for 0h, 4h, 12h, 24h, 31h, or 48h of treatment and subsequently, the roots were harvested. For lateral root induction, in vitro grown 3-day-old *M. truncatula* J5 plants of which not-induced control samples separated were transferred to new plates containing 150 µM N-1-naphthylphtalamic acid (NPA, Greyhound Chem Service, Birkenhead, UK) for 2 days (NPA control sample), after which lateral roots were induced by transferring them to plates containing 10 µM NAA for 12h, 24h, 48h, 72h or 4 days.

**Agrobacterium rhizogenes-mediated transformation**

Approximately 30h after germination, when seedling radicle length was around 10 mm, the radicle was sectioned at approximately 3 mm from the root tip with a sterile scalpel. Sectioned radicles were inoculated by coating the freshly cut surface with A. rhizogenes A4TC24 containing the introduced binary plasmid. The A4TC24 strain (derived from A4T strain; Quandt and Hynes, 1993) was grown on solid YEB medium with appropriate
antibiotics. The inoculated sectioned seedlings were incubated on Kalys agar containing SOLi medium supplemented with 1 mM NH₄NO₃ in square petri dishes which were vertically placed in a growth chamber at 20° C for 1 week (16h photoperiod and a light intensity of 70 µE per s m⁻²), and then transferred to a 25° C growth chamber with identical light conditions. Hairy roots emerging from the radicle section were first observed approximately 1 week after A. rhizogenes infection (Boisson-Dernier et al., 2001). After one month, the chimeric plants were transferred to perlite and inoculated with S. meliloti 1021 (OD₆₀₀ 0.4, resuspended in SOLi). Screening for GFP expressing cotransformed hairy roots was performed using a stereo microscope MZFLII (Leica) equipped with a blue-light source and a Leica GFP Plus filter set.

**Sequence identification and constructs**

The AtNAC1 coding sequence (Genbank Acc. N° AF198054) was hybridized against the M. truncatula Gene Index database (MtGI release 8.0; [www.tigr.org/tdb/tgi](http://www.tigr.org/tdb/tgi)) by TBlastX analysis. DNA sequence data were assembled and analyzed using the GCG package (Genetics Computer Group, Madison, WI). Identity and similarity percentages at the amino acid level were determined by using the GAP program and alignments were produced by ClustalW analysis in GCG. Nuclear localization signal sequence (NLS) prediction was obtained by PSORT analysis of the protein sequences.

Primer sequences used for amplification of the ORFs, 3' UTR RNAi regions, in situ hybridization probes, and RT-PCR and qRT-PCR are shown in Table 1. qRT-PCR primers were designed with the Beacon Designer 4 Program (Biorad). To obtain correct amplification products, all PCR reactions were performed using Pfx proofreading or High-fidelity (Hifi) Taq DNA polymerase (Invitrogen, Life technologies, USA).

PCR fragments corresponding to the full-length open reading frames (ORF) of MtNAC1, MtNAC2 and MtNAC3 were cloned in the pB7GW2D binary vector ([www.psb.ugent.be/gateway](http://www.psb.ugent.be/gateway)). RNAi constructs were obtained by cloning the amplified gene-specific 3'UTR fragments for MtNAC1, MtNAC2 and MtNAC3 in the binary pK7GW1WG2D vector (Karimi et al., 2002). T-DNA regions of both binary vectors contain the CaMV 35S promoter (De-Loose et al., 1995) for expression of the inserted sequences and an additional egfp selectable marker (Haseloff et al., 1999) driven by the constitutive rolD promoter (Goddijn et al., 1993). The binary vectors were introduced into A. rhizogenes A4TC24 by electroporation and transformants were selected with spectinomycin.
For in situ hybridization and southern probes, amplified fragments (see Table 1) were cloned in the pGEM-T Easy vector according to the manufacturer’s protocol (Promega, Mannheim, Germany).

DNA sequencing was carried out with universal SP6 and T7 primers or with the pDONR221FW and pDONR221REV primers, for identification of the 3’UTR or ORF sequences, respectively.

The MtNAC1 1500 bp promoter sequence was isolated through sequential nested PCR amplifications from genomic DNA of M. truncatula Jemalong J5 which was prepared according to a small-scale modified version (D. Barker, LIPM, INRA-CNRS, Toulouse, personal communication) of the protocol used in Dellaporta et al. (1993). By using several primer sequences, sequential rounds of genome walking were performed using the BD GenomeWalker™ Universal Kit as described in the manufacturer’s protocol (BD, Biosciences Clontech, Palo Alto, CA).

<table>
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<th>Gene</th>
<th>Experiment</th>
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<th>Reverse primer</th>
<th>Product length</th>
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TABLE 1: Primer sequences used for PCR reactions to design the probe fragments and overexpression and RNAI constructs, and used for Q-PCR analysis on the M. truncatula NAC1-like genes examined in this study. The MENOD40 and MrRP1 genes were used as a control for nodulation in the Q-PCR and RT-PCR experiments resp., and the MELF1alpha gene as a constitutive control. (*), PCR product was used for amplification with primers with in addition to the gene-specific region the attB1 and attB2 sequences necessary for cloning in the Gateway pDONR221 vector according to the Invitrogen manufacturer’s protocol.
Southern analysis

M. truncatula genomic DNA was extracted from young leaves using the Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer’s protocol. 10 µg was digested with EcoRV, HindIII and HincII restriction enzymes according to standard protocols (Sambrook et al., 1989) and were separated on a 1 % agarose gel. The DNA was transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany) and hybridized with the P32 radio-active labeled probes at 60° C for 1h in hybridization buffer (5xSSC, 5x Denhardt, 0.5 % SDS, salmon-sperm DNA). Probes were generated using the primers shown in Table 1 and the pGEM-T Easy-MtNAC1, -MtNAC2, or -MtNAC3 as template sequences. Radio-active labeling of the purified PCR products was performed with the Rediprime II Random Prime Labelling System (Amersham Biosciences). After exposure, the membranes were analyzed with a PhosphorImager (Amersham Biosciences).

RT-PCR and quantitative RT-PCR

Total RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 2 µg of total RNA using the Superscript RT II kit (Invitrogen) and oligo(dT)18, according to the manufacturer’s instructions. For RT-PCR, the resulting single strand cDNA was diluted to 200 µl, 1/20 of this volume (10 µl) was used in a 20 µl PCR sample further consisting of 2 µl 10 x PCR buffer (Boehringer), 0.5 µl of 10 mM dNTPs, 1 unit Taq DNA Polymerase (Boehringer), and 10 pmoles of sense and antisense primers (Table 1). The program consisted of 20 cycles of amplification for 30 s at 94° C, 30 s at 55° C, and 30 s at 72° C. Reverse transcription (RT)-PCR analysis was performed as described by Corich et al. (1998). PCR products were detected by autoradiography after blotting to a Hybond-N nylon membrane (Amersham Biosciences, Little Chalfont, UK) as in Corich et al. (1998). Probes were generated from the purified PCR product with the Rediprime II Random Prime Labelling System (Amersham Biosciences) and the membranes were analyzed with a PhosphorImager (Amersham Biosciences). RT-PCR analysis was repeated twice and results were quantified to the constitutive control with ImageQuant software.

For qRT-PCR, the first strand cDNA was diluted to 1 ml and 10 µl was used per reaction. qRT-PCR was performed as described in Vlieghe et al. (2005), with the iCycler iQ (Bio-Rad, Hercules, CA) using 200 nM primers and Platinum SYBR Green Supermix-UDG (2x) (Eurogentec, Seraing, Belgium) supplemented with fluorescein dye in a final volume of 25 µl.
per reaction, according to the manufacturer’s instructions. Relative expression was calculated as the ratio of normalized gene expression against the constitutively expressed MtELF1alfa elongation factor 1 (Table 1), by using the $2^{\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Reactions were done in triplicate and averaged. Primers used (Table 1) had a calculated melting temperature of $59^\circ$C $\pm$ 2 and were unique as predicted by BlastN against the MTGI (TIGR) and the Medicago EST Navigation System database (Journet et al., 2002), and was confirmed by sequencing of the amplification products. qRT-PCR analysis was repeated on 3 biological repeats.

**In situ hybridization**

10 µm sections of paraffin-embedded (un)inoculated roots and nodules were hybridized in situ as described by Goormachtig et al. 1997. $^{35}$S-labeled antisense and sense probes were produced according to standard procedures (Sambrook et al. 1989). Plasmids pGEM-T Easy-MtNAC1, -MtNAC2, -MtNAC3 were digested with Sall and SacI to yield a linear template for in vitro transcription with T7 and SP6 RNA polymerase (Invitrogen), respectively. The in situ hybridization of MtNAC1 transcripts was performed twice. Hybridizing sense probes did not yield signal above background, and MtRIP1 was used as a positive control for signal during the early nodulation stages (data not shown).

**Bright- and dark-field microscopy**

Microscopy was performed as described by D’Haeze et al. (1998). For light microscopy, nodules were fixed in 2.5% gluteraldehyde in 0.05 M cacodylate buffer pH 7.0, washed, dehydrated and embedded in Technovit 7100 (Kulzer Histo-Technik, Wehrheim, Germany) according to the manufacturer’s instructions. Sections of 5 µm were cut on a microtome (Reichert-Jung, Nussloch, Germany), mounted on Vectabond-coated slides (Sigma-Aldrich, St. Louis, MO), and stained with 0.5 % toluidine blue (Van de Velde et al., 2003). After further mounting of the slides with Depex (Sigma-Aldrich), they were examined with a bright- and/or dark-field microscope (Leica DMBL). Images were taken with an Axiocam digital camera (Zeiss) and processed with corresponding software (Axiovision).

**Histochemical localization of β-glucuronidase activity**

GUS assays on MtNAC1-promoter-GUS containing transgenic roots were done as described previously (Van de Eede et al., 1992). The presence of blue cells was analyzed with a stereo microscope MZFLIII (Leica).
ACKNOWLEDGMENTS

I would like to thank Annick De Keyser and Christa Verplancke for technical assistance, Karen Migneaux for performing the promoter-gus analysis and providing the lateral root induction series and Katrien D’Haeseleer for contributing to the RT-PCR analysis, in situ hybridization, cloning and hairy root transformation (undergraduate thesis).
CHAPTER 7

CLE peptide signaling in *Medicago truncatula*
INTRODUCTION

Plant development is controlled by phytohormone landscapes which are dynamic in time and space. The hormones auxin, cytokinin, abscisic acid, gibberellin and ethylene are synthesized, transported, stored and degraded throughout the plant’s different developmental stages. Multiple interactions between these signaling compounds and the more recently studied brassinosteroids, jasmonate (JA) and salicylic acid govern organ patterning, meristem initiation, cellular differentiation and integrated responses to the environment. Also peptides play a role as signaling molecules in plants. The first one identified, systemin, functions in local and systemic wound responses (Pearce et al., 1991; Wasternack et al., 2006). This 18-amino acid peptide is derived from the C-terminus of a secreted precursor protein prosystemin (McGurl et al., 1992) and acts at very low concentrations in a structure-dependent way. The presence of systemin in wound exudates of tomato (Lycopersicon esculentum) was sufficient to systemically induce proteinase inhibitor (PI) genes and an antisense prosystemin construct impaired systemic wound response in transgenic tomato (McGurl et al., 1994). Systemin orthologs have been identified in closely related Solanaceous species, such as potato, black nightshade and bell pepper (Constabel et al., 1998), but not in Nicotiana tabacum or in A. thaliana (Ryan et al., 2002). The tomato gene for prosystemin is expressed exclusively in phloem parenchyma cells and is induced by wounding and by JA (Narvaez-Vasquez and Ryan, 2004). After processing of the prosystemin, the systemin peptide binds a leucine-rich repeat receptor-like kinase (LRR-RLK), the SR160 protein (Scheer and Ryan, 2002), the octadecanoid pathway for JA synthesis is activated and a systemic response is established (Li et al., 2002). Systemin-induced JA or related compounds are presumably the systemic signal in the wound response (Schilmiller and Howe, 2005).

Another example of peptide signalling is via the pentapeptide phytosulfokine (PSK), which was first isolated from asparagus cell suspension cultures (Matsubayashi, 1997) and from rice (Yang et al. 1999), and which is widely distributed in higher plants. Again, the peptide is derived from the C-terminus of a precursor polypeptide which might be secreted due to the presence of a putative signal peptide at the N-terminus (Bisseling, 1999; Matsubayashi, 2001). The synthesis of PSKs is dependent on cytokinin and on auxin and, together with these hormones PSKs promote dedifferentiation and cell division at nanomolar concentrations. The peptide is perceived by an LRR-RLK receptor protein (Matsubayashi et al., 2002) and stimulates tracheary element differentiation in Zinnia mesophyl cells (Matsubayashi et al., 1999) and somatic embryogenesis in carrot (Kobayashi et al., 1999).
Shoot apical meristem (SAM) formation in Arabidopsis thaliana involves the action of the CLAVATA3 peptide (Fletcher et al., 1999). The WUSCHEL (WUS) homeodomain transcription factor is specifically expressed in the organizing centre of the SAM (Mayer et al., 1998). WUS confers stem cell identity to the overlying neighbouring cells through an as yet unknown signal and induces expression of the CLV3 gene (Figure 1). CLV3 encodes a 96 aa secreted polypeptide that interacts with the CLV1/CLV2 heterodimeric receptor complex (Rojo et al., 2002). CLV1 is a leucine-rich repeat (LRR)-receptor kinase and CLV2 is a LRR-receptor protein (Trotochaud et al., 1999, 2000). Activation of the CLV1/CLV2 receptor by CLV3 downregulates WUS expression and confines the size of the organizing centre (Schoof et al., 2000; Lenhard and Laux, 2003). A. thaliana CLV3 mutants show an expanded SAM, fasciation of stems and leaves, and supernumerary floral organs (Clark et al., 1995), while ectopic expression of CLV3 results in plants in which the SAM is not maintained and organogenesis from the shoot tip is arrested (Brand et al., 2000). A Kinase Associated Protein Phosphatase (KAPP) and POLTERGEIST (POL) type 2C phosphatase negatively regulate CLV-signaling through interaction with CLV1 (Trotochaud et al., 1999; Yu et al., 2003). Other A. thaliana CLV1-related receptor-like kinases (RLKs) involved in SAM functioning, are the three BAM (derived from 'barely any meristem') receptors for which opposite roles to that of CLV1 are suggested because BAM knock-out mutants show a loss of the stem cells at the SAM and floral meristem (DeYoung et al., 2006). The BAM receptor genes have broad expression patterns, indicating multiple developmental roles for BAM receptors, such as the control of leaf shape, size, and symmetry, but also male gametophyte development and ovule specification (DeYoung et al., 2006). Presumably the BAM receptors function like CLV1, by interaction with a peptide (DeYoung et al., 2006).

In A. thaliana 31 putative CLV3/ESR (CLE)-related peptides have been identified that share a conserved C-terminal region with CLV3 and with maize ESR peptides (Cock and McCormick, 2001; Rojo et al., 2002; Sharma et al., 2003; Strabala et al., 2006).
encoded CLE peptides were shown to be secreted (Cock and McCormick, 2001; Rojo et al., 2002; Sharma et al., 2003) and ectopic expression of CLV3 and of 17 CLE genes resulted in pleiotropic phenotypes, suggesting a very diverse panel of functions for CLE peptide signaling in organ size regulation, apical dominance and root growth (Strabala et al., 2006). Applying 14-amino acid synthetic peptides to the root resulted in consumption of the root meristem, proving the biological activity of the C-terminal CLE motif (Fiers et al., 2005). Presumably, in vivo a proteolytic cleavage releases the active CLE peptide as shown for the CLV3 peptide (Ni and Clark, 2006).

Based on overexpression phenotypes, the 17 CLE peptides have been divided in 4 groups, the WUS mutant-like, the dwarf, the shrub-like and long-root CLE’s. For each group, a consensus CLE sequence and typical amino acids have been determined (Strabala et al., 2006). Moreover, the hallmark C-terminal PLH motif of the CLE-box might be important for the consumption of the root meristem (Fiers et al., 2005; R. Whitford, personal communication). The CLE peptide overexpression phenotypes and the sequence similarity in the CLE domain suggest the occurrence of functional redundancy.

The genetic interaction of CLV3 with the CLV1/CLV2 cell surface-based receptor complex shows strong similarity to peptide hormone signaling in mammalian systems. In these systems, peptide prohormones are synthesized, varying in size and organization, and are classified into families based on their structure and sequence homology (Rehfeld et al., 1993). After the removal of the signal peptide of the preprohormone, the mature bioactive peptides are proteolytically cleaved from the intermediate prohormone. Although similarities in the processing of peptide hormones between plant and animals are becoming more evident, there exist large differences at the level of the type of receptors that perceive these peptide signals. Plant genomes have an abundance of the Pelle family of receptor-like kinases (RLK/Pelle). The plant RLK/Pelle family exceeds 600 members whilst plasmodium and animals contain less than six with none being identified in fungi to date (Shi and Bleecker, 2003). This plant RLK family was divided into two broad categories by Shi and Bleecker (2001), one is involved in plant growth and development and the other in plant-microbe interactions and defense responses (Becraft et al., 2002). CLV1 is an RLK that belongs to the plant development category. RLKs involved in plant-pathogen interactions include the rice Xa21 important for resistance to a bacterial pathogen (Song et al., 1995), the Arabidopsis FLS2 for flagellin perception (Gomez-Gomez and Boller, 2000), and the tomato (Lycopersicon esculentum) SR160 for systemin perception of wound signaling. A recent study showed that the same LRR-RLK has been isolated as the brassinosteroid
Chapter 7

receptor (BRI1) (Montoya et al., 2002), suggesting a dual role in perception of the peptide hormone systemin and steroid hormones. This observation suggests that developmental RLKs can also be involved in the plant defense responses (Scheer and Ryan, 1999, Scheer and Ryan, 2002; Montoya et al., 2002; Scheer et al., 2003) and suggests additional roles for peptide ligands, such as the CLE peptides, besides functions in plant development.

In addition to endogenous plant peptide signals as ligands for RLKs involved in development and defense responses, exogenous peptide ligands derived from pathogens have been described. Avirulence peptide signals such as Avr4 and Avr9 from Cladosporium fulvum trigger the plant-pathogen HR response in tomato (Joosten and De Wit, 1999). Recently a CLE-peptide was found to be secreted by the oesophageal glands of the cyst-nematode Heterodera schatii (Wang et al., 2001; Olsen & Skriver, 2003) and shown to function in plants (Wang et al., 2005). The plant-nematode interaction is an example of parasitism, which is highly detrimental to the plant. Leguminous plants on the other hand have the ability to form beneficial interactions with microbes.

Legumes have the unique capability to engage into a symbiotic relationship with rhizobia, resulting in specialized organs called root nodules. The nodulation process is triggered in a host plant when the lipochitooligosaccharide Nod factor signals produced by the bacteria are recognized by specific LysM-motif-containing RLK receptors present in root hairs (Gage, 2004 Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Two main nodule developmental pathways are discerned among legume plants, resulting in the formation of indeterminate and determinate nodules, which are characterized by a persistent and transiently active meristem, respectively. Root nodules of Medicago truncatula, the model legume for indeterminate nodule development (Cook, 1999), are characterized by a longitudinal organization of zones whereby the apical meristem (zone I) delivers new cells to the underlying zones (Vasse et al., 1990). In the infection zone (zone II), host cells are infected by the microsymbiont and undergo several rounds of endoreduplication while in the fixation zone (zone III) both the bacteria and the plant cells reach their final differentiation stage during which nitrogen fixation takes place. The proximal senescence zone marks the end of the symbiosis and is characterized by a consecutive degradation of symbiosomes and host cells, coupled to defense responses and nutrient remobilization (Van de Velde et al., 2006).

Little is known about the nodule meristem formation and maintenance or whether genes that are involved in SAM and RAM homeostasis also function in maintaining the balance between proliferation and differentiation within nodule meristems. It is known that
phytohormones have major influences on the development of nodules, so the question rises whether peptide signaling also affects nodulation. A first indication is provided by the early nodulin (enod) gene ENOD40 that is expressed at an early stage in root nodule organogenesis and only encodes short ORFs. Overexpression of the M. truncatula ENOD40 gene and transient expression of the ENOD40 region spanning the oligopeptide sequence induces cortical cell division and increased nodule primordium initiation in roots (Charon et al., 1997, 1999). Whether ENOD40 functions as RNA or as a peptide is still not clear. In vitro translation of the conserved ORFs might indicate that indeed ENOD40, at least in part, codes for peptide signals (Sousa et al., 2001; Rohrig et al., 2002).

A second indication comes from a putative receptor complex, a CLV1-like LRR-RLK, that is involved in autoregulation of nodulation (AON) (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Penmetsa et al., 2003; Schnabel et al., 2005). The number of nodules that form on a legume root is controlled through AON, a process that is nitrate sensitive. AON acts on nodule primordium formation in a shoot-dependent regulatory mechanism. The hypernodulating HAR1, NARK and SUNN mutants of L. japonicus, G. max and M. truncatula, respectively, have an increased number of nodules due to a defective shoot control of the long-distance signaling during AON (Wopereis et al., 2000; Searle et al., 2002; Nishimura et al., 2002; Penmetsa et al., 2003). An altered root phenotype (more lateral roots and a shorter root) was observed in HAR1 and NARK mutants in the absence of rhizobia, indicating a link between AON and the control of lateral root number (Gresshoff, 2003). In the M. truncatula SUNN mutant (SUNN is an orthologue of HAR1), the short root is not accompanied by an increased lateral root number (Penmetsa et al., 2003). The HAR1, NARK and SUNN genes are the closest legume sequence-relatives of AtCLV1 identified so far. Hence, the question rises whether a CLV3-like interactor contributes to the AON signaling between the root and the shoot.

Screening of the M. truncatula sequences (containing genomic information and EST data) for CLE motifs resulted in the identification of twenty putative M. truncatula CLE genes. The MtCLE genes are expressed in a range of tissues and some are differentially expressed during nodule development. Two MtCLE genes were ectopically expressed in transgenic roots to assay biological activity and a role in root or nodule development. Preliminary results indicate that MtCLE genes are likely to play an important role in M. truncatula root and nodule development.
RESULTS AND DISCUSSION

Identification of M. truncatula CLE genes

In their study of CLV3/ESR-like (CLE) peptides in Arabidopsis, Cock and McCormick (2001) identified five putative Medicago truncatula CLE genes. The substantial increase in M. truncatula EST and genomic sequence resources over the last years (Journet et al., 2002; Lamblin et al., 2003; Young et al., 2005) justified a novel search as a basis to study the role of CLE peptide signaling in a model legume symbiosis. To refine the tBLASTn search, the PAM30 tBLASTn algorithm was used specifically to identify short peptide sequences homologous to the CLE-box (RXXPXXPXXPXXH). An additional selection for ORFs smaller than 450 bp and a selection for the presence of an N-terminal signal peptide were included. Iterative tBLASTn searches of the M. truncatula Gene Index database (MtGI; www.tigr.org) resulted in ten hits displaying the C-terminal CLE motif, half of them corresponding to the previously identified CLE genes (Cock and McCormick, 2001). The ten hits corresponded to six tentative consensus (TC) sequences and four singletons, only two of which were annotated as CLE gene homologues in the TIGR MtGI index (MtCLE5 and MtCLE6; Table1). For these ten hits, an ORF sequence was defined; nine ORFS showed an N-terminal signal peptide as predicted by HMM signalP and neural networks (Bendtsen et al., 2004). Iterative tBLASTn searches of genomic sequence data, using the different C-terminal CLE motifs obtained from the EST resources, resulted in nine additional hits, corresponding to nine ORFs, all of them predicted to have an N-terminal signal peptide and lacking introns, but of which one might be a pseudogene because of the presence of 2 stop codons in its ORF. In addition, MTCLE14 codes for an ORF of 221 amino acids possessing an N-terminal signal peptide and containing 7 tandemly-arranged CLE-boxes, thereby representing a unique CLE peptide structure. Rice (R. Whitford & P. Hilson, unpublished results) and wheat (Olsen and Skriver, 2003) are the only species in which similar multi-CLE motif containing genes have been found.

To summarize, using the available sequence data for M. truncatula a total of 20 hits were obtained that showed a conserved CLE motif. The ORFs varied in length between 156 and 663 bps and the encoded peptides were predicted to have an N-terminal signal peptide, except for MtCLE3 (Table1). The majority of the MtCLE genes have the characteristic RXXPXXPXXH signature. Similar to the Arabidopsis CLE gene family, a high level of sequence divergence is observed outside the CLE motif (Figure 2).
<table>
<thead>
<tr>
<th>CLE gene</th>
<th>BAC</th>
<th>Conserved Domain</th>
<th>Genomic Sequence</th>
<th>Complete CDS</th>
<th>SP</th>
<th>Length (aa)</th>
<th>EST (Gebank Acc No*)</th>
<th>Expression</th>
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<td>Y</td>
<td>Y</td>
<td>124</td>
<td>AW586793, BQ139113</td>
<td>Root - Glomus versiforme Immoculated, Leaf - Phoma medicaginis Immoculated</td>
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<td>Y</td>
<td>Y</td>
<td>102</td>
<td>BI311733</td>
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<tr>
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<td>-</td>
<td>RKPSPCPOPLHNN</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>45</td>
<td>BQ157494</td>
<td>Seedlings – Gamma Irradiated</td>
</tr>
<tr>
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<td>RGVPSSANPLHNN</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>66</td>
<td>AW329414</td>
<td>Root – Phosphate Starved</td>
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<tr>
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<td>84</td>
<td>BM812815, BE999212</td>
<td>Root – Nematode Infected Senescent Root Nodules</td>
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<tr>
<td>MtCLE6</td>
<td>-</td>
<td>HEVPSGPNIPSN</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>99</td>
<td>BF650504, BE9-1833, CX518468</td>
<td>Root derived cell culture Roots – beta-glucan elicited AMV infected leaves</td>
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<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>86</td>
<td>-</td>
<td>Methyl Jasmonate-Elicited Root Cell Suspension Culture</td>
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<td>Y</td>
<td>120</td>
<td>CX530269, CX530352</td>
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<td>Y</td>
<td>139</td>
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<td>-</td>
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<td>101</td>
<td>-</td>
<td>-</td>
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<td>Y</td>
<td>72</td>
<td>-</td>
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<td>RQVSPGPDPLHNN</td>
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<td>Y</td>
<td>61</td>
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</table>
Chapter 7

Transcriptional profiling of MtCLE genes

Twenty putative CLE peptides were identified from the available M. truncatula genome sequence. Using quantitative reverse transcriptase PCR (QRT-PCR) fourteen of the 20 MtCLE genes were transcriptionally profiled (no suitable primer pairs could be designed for the remaining six) to determine tissue or organ specific expression and to investigate differential expression during the legume-specific process of nodule development. An overview of the results of all experiments is presented in Figure 3 and 4. In a first experimental set up M. truncatula Jemalong J5 plants were grown on medium containing 1mM NH4NO3 and root tips, SAM, first leaves and cotyledons were harvested after 5 days of in vitro growth. In addition leaves and stems from 1 month old plants, as well as flowers from 4 month old
CLE peptide signaling in *Medicago truncatula*
FIGURE 4:
FIGURE 3, 4 and 4 continued: Transcription profiling of MtCLE genes in M. truncatula. Relative changes in gene expression of MtCLE in 5 experiments are grouped in columns and include the analysis of samples taken over a range of tissues grown in the presence of nitrogen (N+ Tissues and N-systemic response), or in the absence of nitrogen or in nodulated plants (N-systemic response), and during the nodulation process, namely on Early nodule development, on several Early nodulation stages, and on specific stages within a nodule. The relative changes in transcript levels in columns 2, 3 and 5 are given as compared to not-inoculated (NI) nitrogen-starved roots with a reference value of 1, whereas in column 4 and 1, the 2dpi root and the root tip samples are used as a reference, resp. SAM, shoot apical meristem; cotyl, cotyledons; Flow, flowers; Nod, 10 dpi nodulated roots; np, nodule primordia; ynod, young nodules; R, not-inoculated nitrogen-starved roots; N1, nitrogen-fixing, round indeterminate nodules without senescence; N2, nitrogen-fixing, more elongated, indeterminate nodules without senescence; ASN1, apical and PSN1, proximal parts (containing the senescing zone) of nodules with small, young green senescence zone (35dpi); ASN2, apical and PSN2, proximal part of nodules with large green senescence zone (61dpi). No nodulation expression data of MtCLE16 are shown for the nodulation-related expression because no amplification was obtained, due to its shoot-specificity. Error bars, standard deviation on the technical triplicates.
plants were analyzed (Figure 3 and 4; Tissues+N). In a second experiment, a range of
tissues was compared to test effects of nitrate or nodulation on MtCLE gene expression
(Figure 3 and 4; N-systemic response). Root tips, the first leaf and the SAM of Jemalong
plants grown in vitro for 5 days in the absence or presence of 1mM NH₄NO₃ were harvested,
as well as leaves, stems and roots of plants grown in perlite for 15 days without nitrate, with
nitrate, or nodulated for another 10 days.

To investigate MtCLE transcript profiles during nodulation, nitrogen-starved roots and
several stages of developing nodules were collected in three independent experiments. From
a first set of M. truncatula plants, non-inoculated zone I regions of the root, S. meliloti
inoculated roots (2 dpi), corresponding to bacterial attachment in zone I of the root, along
with nodule primordia (12dpi) and young fixing nodules (16dpi) were sampled (Figure 3 and
4; early nodule development). A second set of S. meliloti inoculated plants was used for
isolation of tissue in early nodulation stages, including inoculated roots zone I (2dpi), zone I
root tissue containing infection threads (6dpi), nodule primordia (8dpi) and young fixing
nodules (14dpi) (Figure 3 and 4, early nodulation stages).

As a control for S. meliloti induced gene expression MtENOD40 transcript levels were
determined in both developmental series (Figure 5A and B) and found to be 26 and 15 fold
upregulated in 16dpi and 14dpi samples, respectively. To compare MtCLE transcript levels
of different nodule developmental stages, nodules were dissected according to Van de
Velde et al. (2006) (Figure 5C). Root tissue was isolated from nitrogen-starved non-inoculated
plants (R); very young round nitrogen-fixing nodules without senescence zone (N1);
somewhat older elongated nitrogen-fixing nodules, still without senescence zone (N2);
apical parts containing the nodule meristem (ASN1) and proximal parts containing the
senescing zone (PSN1) of 35dpi nodules with incipient senescence zone; apical (ASN2) and
proximal (PSN2) part of 61 dpi nodules with

FIGURE 5: CLE peptide analysis in M. truncatula.
A and B demonstrate the relative MtEnod40 expression levels on the nodule developmental series.
In C, the plant material harvested for transcription profiling within a nodule.
large green senescence zone (Figure 3 and 4, within a nodule).

MtCLE genes are expressed at different levels in a diversity of tissues at different developmental stages.

The MtCLE16 expression profile reveals an outspoken shoot-specific transcript accumulation pattern but the expression is reduced in the SAM and in the 1st leaf (Figure 4). The peptide could play a role in leaf organ development, in the control of leaf size or shape, or in AON. The possibility that MtCLE16 could be a ligand of the SUNN receptor contributing to the AON mechanism (Penmetsa et al., 2003) should be further explored. Such a ligand should be expressed in the upper part of the plant because the signal that controls the root physiology is shoot-derived and SUNN-dependent. Transcript levels could be either constitutive (the MtCLE16 profile is a candidate) or upregulated in nitrogen grown or nodulated plants. The latter transcript profile was not detected in the population that we screened.

MtCLE15 shows a high transcript level in stems and SAM, and transcripts were abundant in nitrate-rich grown and nodulated roots (14 and 5 fold, respectively). Moreover, a dynamic expression was observed during nodulation as transcription was repressed during the early stages of nodule development (Figure 3, Early nodule development and Early nodulation stages), restored to the same level as in uninoculated roots in mature nodules, and strongly enhanced in senescent nodules (ASN2 and PSN2). This pattern might suggest a role in the regulation of nodule numbers, but needs further study.

MtCLE10 transcripts accumulate in flower, leaf, and SAM, are absent in the root tissue and are upregulated during nodule primordium development and in senescent nodule tissue (PSN2), suggesting a potential role in nodulation.

The MtCLE4 gene reveals a root specific expression pattern (transcripts were mainly detected in the root tip) and expression is induced during nodulation. A four fold induction is detected at 6 dpi, and is maintained throughout nodulation with even a 5.8 fold induction in the basal part of mature nodules, where senescence starts (PSN1). The expression in the roots together with the upregulation of MtCLE4 during nodule development led to the hypothesis that this peptide contributes to the determination of root and nodule cell fate and confers “root” identity to nodule development.

MtCLE12 and MtCLE13 are good candidates for regulating nodule meristem homeostasis. MtCLE12 gets very highly upregulated from nodule primordium formation on (> 10 fold at 6dpi; Figure 3 Early nodulation stages; > 40 000 fold in nodulated roots; Figure 3 N-systemic response) and further throughout nodule development. MtCLE12 is also highly
abundant in the SAM (56 fold compared to the root tip; Figure 3 Tissues +N; 1800 fold compared to roots; Figure 3 N-systemic response), suggesting that legumes have indeed recruited shoot-like features for nodule meristem signaling. MtCLE13 transcript level in the SAM is not significantly higher than in the root tip. A relative differential transcript level of more than 430 fold in nodule primordia compared to roots (Figure 3 Early nodule development) is a strong hint that MtCLE13 could be really specific for nodules. As MtCLE12 is already highly expressed at 6dpi, it might function in the installation of the primordium. MtCLE13 only seemed to be highly induced between 8 and 14 dpi and might thus be involved in later steps, such as the differentiation of the nodule meristem. A crucial role in nodule meristem initiation and homeostasis is proposed for MtCLE12 and MtCLE13 peptides and further functional analysis will be performed.

To conclude, a limited set of transcription profiling experiment have provided good indications for a role of MtCLE12, 13, 4, 10, and 15 in M. truncatula nodule development.

**Biological functioning of CLE genes in M. truncatula**

To prove functionality of the MtCLE genes identified in this study, some MtCLE genes were ectopically expressed using the A. rhizogenes-mediated transformation method (Boisson-Dernier et al., 2001) and the root and nodule phenotype was analyzed.

The MtCLE gene overexpression constructs, together with an egfp reporter gene, were introduced in M. truncatula roots. A. rhizogenes infection leads to the outgrowth of cotransformed roots from the infection site, every root representing an independent transformation event. In vivo screening of co-transformed roots is possible through the GFP expressing in the transformed roots. Inoculation of these transgenic roots with S. meliloti allows normal nodule formation.

MtCLE5 and MtCLE14 were selected for overexpression in M. truncatula transgenic roots, a choice based on the similarity of MtCLE5 to the typical WUS mutant-like group (Strabala et al., 2006) and on the intriguing unique structure of MtCLE14. A CaMV 35S and a nodule specific leghemoglobin (pLb1) promoter showing activity in the infected nitrogen-fixing nodule tissue (Carvalho et al., 2003), were used to drive the expression of the MtCLE5 and MtCLE14 in M. truncatula. For each construct, a total of 13 (=n) independent transgenic roots were analyzed for root length (Figure 7A). Root growth was assayed for GFP expression at 20 days after A. rhizogenes infection. Transgenic roots containing the pTC85913-GUS construct were used as a control and contain a T-DNA with an egfp reporter gene and the promoter of the cystein protease encoding gene (derived from TC85913) fused to the gus gene (Van de Velde et al., 2006).
p35S::MtCLE5 transgenic roots are shorter (4 mm) (Figure 6A) than control pTC85913-GUS transgenic roots (28.9 mm) (Figure 6B) (p-value = 7.01E-14). Root initiation and early growth was similar to the controls, however after several days these roots stopped further growth. p35S::MtCLE14 and pLb1::MtCLE5 transgenic roots showed a similar average root length compared to the control (30.3 mm and 28.5 mm, resp.) (p-value = 0.337 and 0.433, resp.) (Figure 7A).

Technovit sectioning and microscopic analysis of six root tips per construct demonstrated that both, p35S::MtCLE5 and p35S::MtCLE14 roots have a shorter elongation zone than pLb1::MtCLE5 and control pTC85913-GUS roots (Figure 6C to F). The root hair zone is closer to root tip and the numbers of toluidine blue stained cytoplasmically dense cells in the meristematic region was lower. Root hairs located within close proximity to the root tip, were observed in all p35S::MtCLE5 transgenic roots (n=13). Collectively, these data suggest that the stem cell population has been consumed as has been observed upon ectopic expression of CLV3-like genes in Arabidopsis (Fiers et al., 2004). Additionally, an average number of 0.02 ± 0.02 lateral roots per p35S::MtCLE5 transgenic root (n=35) was visually scored as compared to 1.09 ± 0.2 for control pTC85913-GUS transgenic roots (n=81) at 20 days after A. rhizogenes infection, suggesting that lateral root initiation might also be perturbed.

These results show that the method of overexpressing potentially MtCLE peptide-encoding genes in M. truncatula roots offers a useful bio-assay for the functionality of these peptides. The root growth effect observed after introduction of p35S::MtCLE5 fits with the observations in A. thaliana as overexpression of genes coding for peptides with very similar
Chapter 7

CLE-boxes resulted in similar phenotypes. The root growth differences between p35S: MtCLE5 and p35S: MtCLE14 suggest that the corresponding genes have different capabilities to trigger endogenous CLV-like receptors involved in root meristem maintenance.

![Figure 7: The average length (in mm) of the transgenic root lines for each construct (n= 13) obtained 20 days after A. rhizogenes infection (A). B, the average number of nodules and of fixing nodules 26 days after inoculation with S. meliloti. n= number of plants analyzed per construct, Error bars, standard errors.](image)

Furthermore, the nodulation capacity of these MtCLE overexpressing roots was analyzed. Because of the severe short root phenotype, p35S: MtCLE5 transgenic roots could not be used for inoculation. Ectopic expression of MtCLE5 in the fixation zone of the nodule was obtained through A. rhizogenes transformation with the pLb1: MtCLE5 construct-containing vector. At 26 days post S. meliloti inoculation (dpi) p35S: CLE14 transgenic roots (n=11) showed on average 1 ± 0.36 nodules (p-value = 0.0038), whereas pLb1: MtCLE5 roots (n=11) had 5.36 ± 1.61 nodules on average (p-value = 0.72) and control roots (n= 15) 4.67 ± 1.04. Analysis of the proportion of nitrogen-fixing nodules, as determined by the presence of leghemoglobin (pink colouration), at 26 dpi showed that p35S: MtCLE14 roots had on average 0.45 ± 0.21 fixing nodules, pLb1: MtCLE5 roots an average of 2.09 ± 0.59 and control roots an average of 2.27 ± 0.56 fixing nodules. Hence, an approximately equal fraction of fixing nodules was observed on pLb1: MtCLE5 roots (40 %), p35S: MtCLE14 roots (45 %), and control roots (48 %). These results were confirmed with a second set of transgenic lines containing the pLb1: MtCLE5 and p35S: MtCLE14 constructs. In conclusion, nodulation of pLb1: MtCLE5 roots was not different compared to nodulation of control roots, whereas inoculation of the p35S: MtCLE14 roots resulted in a significant lower number of nodules formed at 26dpi.

From these preliminary results we concluded that ectopic overexpression of MtCLE5 has no effect on nodulation and taking into account its expression pattern, this peptide is most likely functioning in root development. Ectopic expression of MtCLE14, on the other hand,
CLE peptide signaling in *Medicago truncatula*

results in a reduced amount of nodules developed at 26dpi, providing functional evidence that CLE peptide signaling is involved in the nodule formation process.

Another approach to examine the biological functioning of the CLE peptides was launched by Fiers et al. (2005) through the application of synthetic peptides to *A. thaliana* roots, resulting in root meristem consumption resembling the overexpression of AtCLE19 in *Arabidopsis*. As synthetic *Arabidopsis* CLE19 peptide was available in our laboratory (R. Whitford, pers. comm.), a pilot experiment was performed on *M. truncatula* wild type and sunn roots.

The *M. truncatula* hypernodulating SUNN mutant is mutated in the gene encoding a CLV1-like RLK and is so far the only *M. truncatula* mutant known to be defective in putative CLE-like ligand perception. The SUNN mutant is defective in autoregulation of nodulation, which results in increased nodule numbers, but not in more lateral roots (Penmetsa et al., 2003, Schnabel et al., 2005). The SUNN mutant root phenotype is restricted to a shorter main root which is presumably caused by a shortening of the root cortical cells (Van Noorden et al., 2006), and is controlled through shoot-derived signaling (Schnabel et al., 2005).

Six of each wild type and SUNN mutant plants were grown in vitro in the presence and absence of 10 µM AtCLE19 peptide. After 21 days of growth the main root length was measured. Wild type plants treated with AtCLE19 peptide displayed a shorter root after 21 days of growth (4.85 ± 0.22 cm compared to 6.38 ± 0.46 for untreated wild type roots). The AtCLE19 peptide was shown to cause root meristem consumption upon ectopic expression in *A. thaliana* (Fiers et al., 2004; 2005), so the shorter root length upon AtCLE19 application indicates that the exogenous CLE peptide addition results in root meristem consumption in *M. truncatula* as well, providing evidence for the biological activity of these peptides in *M. truncatula*.

On the other hand, root growth of SUNN mutants was not affected by addition of the AtCLE19 peptide to the roots (4.36 ± 0.55 cm and 4.066 ± 0.33 cm, without and with peptide, respectively). This might indicate a function for SUNN in perception of CLE ligands in the root. Until now, no additional CLV1-RLKs have been identified in *M. truncatula*, although the characterization of RLP1 (Schnabel et al., 2005), which is a truncated copy of the SUNN gene lacking a kinase domain and co-expressed with SUNN, suggests the presence of additional receptor-complexes. Schnabel et al. (2005) predict that RLP1 is not the CLV2 orthologue, because by sequence alignment a closer homologue was identified in
another BAC clone. Thus, the continued search for CLV1/2 orthologues in legumes might result in better candidates.

**CONCLUSIONS AND PERSPECTIVES**

The identification of twenty CLE genes in *M. truncatula* suggests that this family of small peptides functions in legume development. The CLE domain structure is preserved among species and the processing of CLE proteins might result in active MtCLE box peptides.

The expression profiling on a range of tissues and nodule developmental stages revealed diverse expression patterns, including a differential transcript accumulation of several genes during nodule development. This preliminary analysis provides clear information to select MtCLE12, MtCLE13, MtCLE4, MtCLE10, and MtCLE15 genes for further study because of their upregulated expression during nodule development or senescence.

Ectopic expression and knock-down of MtCLE12 and MtCLE13 in transgenic *M. truncatula* roots, as well as insertion or deletion mutant analysis might indicate whether the peptides define nodule specificity to lateral root organs. The expression pattern of MtCLE4 during root development should be tested to confirm a ‘root identity’ role to this MtCLE peptide.

Overexpression might result in root meristem consumption, as observed for MtCLE5; hence a nodule meristem specific promoter is needed for nodulation studies. The MtLb1 promoter is not active in the nodule meristem, but the promoter activity of the endogenous MtCLE12 and MtCLE13 genes potentially involves nodule meristem specificity, which subsequently needs to be analyzed.

Finally, evidence was obtained for the biological activity of CLE peptides in *M. truncatula* via a bio-assay in which MtCLE genes were overexpressed in transgenic *M. truncatula* roots and via in vitro application of CLE peptide to wild type roots. These preliminary results provided evidence for active MtCLE peptide signaling in the legume *M. truncatula* which will be investigated in depth in the near future.

**METHODS**

**Plant Lines, Growth Conditions, and Inoculations**

Growth of *Medicago truncatula* Jemalong J5 and plant inoculation with *Sinorhizobium meliloti* 1021 were performed as described at [www.isv.cnrs](http://www.isv.cnrs).
S. meliloti 1021 cultures were grown at 28° C in YEB (Vervliet et al. 1975).

In silico identification of M. truncatula CLE genes

BLAST searches were performed at The Institute for Genomic Research (TIGR; www.tigr.org/tdb/tgi/) or at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). The CLE family was identified by repetitive searches similar to that conducted by Cock and McCormick (2001). Repetitive searches at the TIGR Medicago Truncatula Gene Index (MTGI Release 8.0) with the TBLASTN PAM30 algorithm were first conducted using the Arabidopsis CLE-box consensus (RXXPXXPXPXH). Confirmation of the first identified sequence to encode a Medicago CLE-like peptide was based on the predicted peptide length (<150 amino acids), the presence of a C-terminally localized CLE-box and an N-terminal signal peptide as predicted by HMM signalP and neural networks (Bendtsen et al., 2004). MtCLE1 (Genbank EST AW586793) was the first sequence identified and was used to repeat the same search, each time with a novel homologous CLE-box sequence. This was done until no new family members were found in the EST data. These CLE-box sequences were then used in the same iterative BLAST searches to identify additional putative CLE peptides from the partially completed genomic sequence. Sequence alignments were made with AlignX within the VectorNTI Advance v.10 suite of programs (http://www.invitrogen.com).

Transcription analysis

Total RNA was isolated using the RNeasy kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). First-strand cDNA synthesis and quantitative PCR were done as described in Vlieghe et al. (2005). The relative expression was calculated by normalization with the gene expression of the constitutively expressed 40S ribosomal protein S8 (tentative consensus EST cluster TC100533 in the MTGI from TIGR) for the series of tissue samples within the nodules (Van de Velde et al., 2006) or the constitutively expressed Translation Elongation factor 1-alpha (TC106485) for the developing nodule and other plant tissue samples, using the 2^ΔΔCT method (Livak et al., 2001). Reactions were done in triplicate and averaged. Primers used (Table 2) had a calculated melting temperature of 59°C ± 2 and were unique in MTGI version 7.0 (TIGR) and the Medicago EST Navigation System database (Jouret et al., 2002).
**Table 2:** Primer sequences used for qRT-PCR on *M. truncatula*.

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>MtCLE5</td>
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**Molecular cloning of MtCLE5 and MtCLE14**

PCR fragments corresponding to the full-length open reading frames of MtCLE5 and MtCLE14 were cloned in pB7GW2D and pKM43GW (www.psb.ugent.be/gateway) for expression under the CaMV 35S (De-Loose et al., 1995) or the pLb1 (D. Barker, INRA, Toulouse, France) promoter, respectively. The presence of the egfp marker (Haseloff et al., 1999) under control of the constitutive rolD promoter (Goddijn et al., 1993) was used as a visible marker for in vivo selection of cotransformed transgenic roots. The binary vectors were introduced in *A. rhizogenes* A4TC24 (derived from A4T strain; Quandt and Hynes, 1993) by electroporation and transformants were selected by spectinomycin resistance.

**Agrobacterium rhizogenes-mediated hairy root transformation**

Approximately 30h after germination, when seedling radicle length was approximately 10 mm, the radicle was sectioned at approximately 3 mm from the root tip with a sterile scalpel. Sectioned radicles were inoculated by coating the freshly cut surface with *A. rhizogenes* A4TC24 containing the introduced plasmid, which was grown on solid YEB medium with the appropriate antibiotics. The inoculated seedlings were placed on Kalys agar (HP 696-7470 Kalys, France) containing SOLi medium (as described at www.isv.cnrs-gif.fr/embo2/manuels/index.html) supplemented with 1 mM NH₄NO₃, in square petri dishes (12 × 12 cm). The petri dishes with the inoculated seedlings were placed vertically in a
CLE peptide signaling in Medicago truncatula

growth chamber at 20° C for 1 week (16h photoperiod and a light intensity of 70 μE per s m$^{-2}$), and subsequently grown at 25° C in identical light conditions. Hairy roots emerging from the radicle section were first observed approximately 1 week after A. rhizogenes inoculation (Boisson-Dernier et al., 2001).

**In vitro application of synthetic peptide to M. truncatula roots**

Two day old seedlings were grown in vitro in square petri dishes (12 × 12 cm) on SOLi medium supplemented with 1 mM NH$_4$NO$_3$ and phosphate buffer (as used in Fiers et al., 2005) or 10 μM AtCLE19 peptide (ServiceXS, Genomax Technologies, Singapore) dissolved in phosphate buffer. The petri dishes containing the inoculated seedlings were placed vertically in a growth chamber at 25° C (16h photoperiod and a light intensity of 70 μE per s m$^{-2}$) and after 23 days of growth, the root length was measured and the amount of lateral roots was counted.

**Microscopic analysis**

Light microscopy analysis was done as described previously (D'Haeze et al., 1998).

**ACKNOWLEDGMENTS**

This study was performed in cooperation with Ryan Whitford (bio-informatics and microscopical analysis of root tips) and Willem Van de Velde (part of transcript profiling and overexpression analysis). I also would like to thank Virginie Mortier and Annick De Keyser for technical assistance.
Perspectives

In this study several aspects of the signaling pathways resulting in the formation of functional nodules were functionally examined. However, further research is required to answer the questions that raised from this research, in particular on the topics that were investigated in Medicago truncula.

The results on the functioning of SINA proteins during nodulation as discussed in chapter 5 look very promising and provide a sound base for further research on the nearly uncharacterized role of this class of E3 ligases in plant ubiquitin-mediated degradation, and moreover on their essential regulatory role during nodulation. We provided evidence that they are important for infection thread growth, during symbiosome differentiation and in nodule senescence. The obvious functions to investigate are the MtSINA genes that were identified and that are good candidates to control sub-programs of nodule development by targeting specific nodulin proteins for proteolytic degradation. More concrete, promoter-gus activity of the MtSINA's will refine their potential point of action during nodulation. Functional analysis of the different MtSINA's by RNAi and ectopic expression might give indications on the specific role of each protein. Another straight-forward experiment includes a yeast two-hybrid screen on early nodulation libraries with the MtSINA2 and MtSINA3 proteins, of which the genes are both upregulated early after inoculation. This would enable the identification of the nodulin genes that are targeted for degradation at the rhizobial infection stage. Even better would be to do a screen on cDNA libraries of each nodulation zone, such as the meristem, infection, fixation and senescence zone of the nodule. In this way, a functional difference of the MtSINA's might be revealed. To prove the essential role of proteasomal degradation action during nodulation, the translational inhibitor MG132 should be used, which might also reveal whether the isolated potential target proteins are subject to proteasomal degradation during nodulation.

In chapter 6, the expression and functional analysis of MtNAC1 reveals an intriguing role for this transcription factor during nodule development. The expression analysis needs to be completed by analyzing the promoter-gus activity on the very early stages of nodulation in transgenic roots using a dsred-expressing rhizobium strain. The functional analysis on RNAi and ectopically expressing transgenic roots provided a reliable indication that modulation of MtNAC1 expression hampers nodulation. However, rhizobial infection could take place and ITs were initiated, although the infection progressed in a disorganized manner. To analyze the morphology of the infection threads in the MtNAC1 overexpressing nodules, electron microscopy will be performed. Furthermore, we recently obtained stable
transgenic R108 lines ectopically expressing and knocking down MtNAC1. A detailed characterization of the nodule phenotype will be performed on these lines. The nod phenotype should be screened for very early nodulation events such as root hair curling and infection thread initiation to find out whether it is the infection process or the primordium formation that is blocked or hampered, or whether the signaling to synchronize these processes is regulated by MtNAC1. By applying cytokinin and auxin to the nodulated transgenic plants, we could analyze the involvement of these hormones in the MtNAC1-regulated signaling during nodulation. Possibly the ectopic root formation on the hairy roots is stimulated through the high auxin balance in these roots, hence stable transgenics might form these ectopic roots after the application of auxin. Another way to test the influence of MtNAC1 on cytokinin and auxin hormone balances is to introduce the promoter ARR5-gus and DR5-gus constructs in transgenic roots of the plants ectopically expressing and knocking down MtNAC1 in comparison to the gus-pattern observed in wild type.

Another intriguing question is what the upstream signals are that trigger MtNAC1 during nodulation. Several mutants defective in the specific nodulation stages will be analyzed for MtNAC1 expression. The hcl mutant, which is mutated in the LYK3 LysM domain-containing receptor and which functions in the bacterial uptake as well as in distant cortical signaling during nodule initiation forms a good candidate to act on MtNAC1 regulation. Also the DMI3 gain-of-function mutant, which produces spontaneous nodules and functions downstream of DMI2 and Ca^{2+}-spiking, will be analyzed. Other interesting candidates are the nodulation-pathway specific NSP1 and NSP2 GRAS transcription factor mutant lines. In addition, it would be interesting to know whether purified Nod factors are sufficient for MtNAC1 induction. Further future experiments might involve the isolation of the downstream targets of MtNAC1. This could be done by an affymetrix-based transcript profiling on the mutant RNAi lines compared to wild type and/or to ectopic expressing lines. However, the relevance of using the 35S promoter in these lines should be reconsidered. The detailed phenotypic analysis of the transgenic plants might indicate whether the 35S expression also influences other plant developmental processes besides nodulation.

The other two NAC genes characterized in this study might also play a role during nodule functioning, although their potential role did not become very clear in this study. Promoter-gus analysis should be performed to determine whether the expression is located in the vascular tissue of the root at specific positions, such as at the nodulation initiation sites. Other techniques might be required to distinguish the transcript location in specific cell types of the root xylem or phloem. A more precise localization might be acquired with
translational gfp-fusion constructs driven by the endogenous promoter. Finally, to obtain evidence for a nodule-related role, stable transgenics might be analyzed.

Last, the identification of MtCLE gene family members by in silico and transcript profiling analysis resulted in a rather obvious selection of the MtCLE12, MtCLE13, MtCLE4, MtCLE10, and MtCLE15 genes for further study because of their upregulated expression during nodule development or senescence (Chapter 7). Hence, a detailed expression analysis by performing promoter-gus and in situ hybridization experiments would be very informative. In addition, it would be interesting to localize the peptides themselves during nodulation. Functionality of CLE genes was proven in M. truncatula roots, suggesting that functional analysis of MtCLE genes might reveal their role in nodulation. Ectopic expression and knock-down of MtCLE12 and MtCLE13 in transgenic M. truncatula roots, as well as insertion or deletion mutant analysis might indicate whether the peptides define nodule specificity to lateral root organs. Overexpression might result in root meristem consumption, hence a nodule meristem specific promoter is needed for nodulation studies, for which the promoters of MtCLE12 and MtCLE13 form good candidates, which will be analyzed. Finally, these peptides form ligands for CLV1-like receptors, so what are the receptors? BAM-like homologues are suitable candidates, and will be searched for in the M. truncatula genomic databases. Expression analysis of these receptor genes might indicate a specific interaction with some of the identified MtCLE peptides involved in nodulation.
SUMMARY/SAMENVATTING
**COVER:** Bright-field microscopy on sections of a 25 dpi M. truncatula mature fixing nodule showing in close-up the differentiated bacteroids that reside in the infected plant cells of the fixation zone.
Plants differ in the response to their environment. The micro-organisms present in the rhizosphere participate in the elicitation of diverse plant responses, which may lead to plant damage and disease through a pathogenic interaction, but also to a symbiosis in which both partners profit from each other's presence. The vast majority of land plants are competent to symbiotically interact with fungi, resulting in arbuscular mycorrhiza on the roots, thereby improving the plant's phosphorous uptake from the soil to advance growth and development.

Legume plants are unique in their ability to engage a symbiotic interaction with Rhizobium bacteria, resulting in the development of new organs on the roots, the nodules. The bacteria that reside in these nodules reduce atmospheric nitrogen to ammonium that can be assimilated by the plant, thereby providing an unlimited source of nitrogen, an essential nutrient for growth in natural ecosystems and agricultural systems. Legumes can be applied as 'green manure' in cropping and in addition provide one third of all the human needs for dietary protein nitrogen due to the production of nutritious protein-rich seeds (Graham and Vance, 2003).

To gain a better insight in the process of symbiotic nitrogen fixation and nodulation, extensive research on the molecular level is performed in many laboratories all over the world and may lead to a range of applications with potential economical and agricultural benefits. Legumes with agricultural importance, such as soybean, pea, bean, alfalfa, etc. are not well studied on a molecular basis because of their large polyploid genomes, recalcitrance for transformation, plant size and long generation periods. Therefore, the research community has selected two plants, Medicago truncatula and Lotus japonicus, as model systems to study the indeterminate and determinate nodulation processes, respectively, but also to improve grain legume cropping by extending the nodule's lifetime and by increasing pod-filling capacities (Cook, 1999; Handberg and Stougaard, 1992). Comparative genome and syntenic studies allow to extend this knowledge to the agriculturally important crops (Young et al., 2003) and might eventually enable nodulation of non-legume crops (Chapter 1). The transformability of the model legumes creates the possibility to study transgenic plants with modulated gene expression, whereas many other legume species are known to be recalcitrant, of which Sesbania rostrata is an example. However, this tropical legume discloses other attractive features and has been extensively studied in our research group.

S. rostrata grows in flooded areas of the Sahel region (Duhoux and Dreyfus, 1982) and can be nodulated by the microsymbiont Azorhizobium caulinodans. The environmental conditions determine the invasion and nodule type, aerated conditions allow root hair curling.
Summary

while submerged plants nodulate through intercellular crack entry invasion at lateral root bases (LRB) or adventitious root bases on the stem (Goormachtig et al., 2004). The versatile nodulation features of S.rostrata are primarily acquired by the altered response to ethylene and gibberellins (D’Haeze et al., 2003; Lievens et al., 2005; for an overview see Chapter 2).

Functional analysis of one of the genes isolated through a differential display experiment, Srchi24, was performed and is described in Chapter 3. Srchi24 codes for a chitinase-like protein that lacks hydrolytic activity, but achieved chitin binding capacity. The similar structure of chitin and the Nod Factors, the bacterial signal molecules which trigger the nodulation process in the plant, suggested a key role for Srchi24 in the perception or scavenging of NFs to guide them to the receptor(s) in the epidermis. Srchi24 shows a basal expression in the root and an early nodulation-related expression in the outer cortical cellayers and around the infection pockets (Goormachtig et al., 2001). Cell wall localization and chitin-binding was shown for in planta produced Srchi24, but no evidence for NF-binding was obtained. S. rostrata knock-down/out transgenic roots showed no difference in the LRB nodulation capacity. However, as chitinase-like proteins have also been linked to developmental functions (Dyachok et al., 2002), the basal expression of Srchi24 in roots together with a reduced root development of transgenic roots containing an Srchi24 RNAi construct might still point to a more general role in organ development (Chapter 3).

While A. rhizogenes transformation of S. rostrata is a valuable and efficient method to obtain transgenic roots, A. tumefaciens transformation was never successful (Vlachova et al., 1987) but would allow to study its synchronous nodulation on the predetermined adventitious root primordia on the stem in transgenic plants. By using a promising new method, the multi-auto-transformation vector system (Ebinuma et al., 1997a), transgenic S. rostrata shoots could indeed be obtained. We showed that S. rostrata is transformable, although regeneration of the transformed tissue was not sufficiently efficient to apply this method in further research (Chapter 4).

Nodules are, like lateral roots, post-embryonically formed on the root (Casimiro et al., 2003; Kondorosi et al., 2005). The formation of both organs depicts several similarities, such as the position opposite the protoxylem poles, a local auxin requirement for their initiation, re-activation of the cell-cycle in the pericycle and/or the cortex, the development of a new vasculature and meristem, and a common autoregulatory mechanism to control the organ number (Mathesius et al., 1998; de Billy et al., 2001; Nishimura et al., 2002; Kondorosi et al., 2005). Hence, the nodulation process most likely recruited several aspects
from the lateral root developmental pathway. Lateral root formation is extensively studied in Arabidopsis thaliana, and the knowledge achieved through A. thaliana research provides a useful base to discover genes specifically functioning in nodulation that have been recruited from the lateral root developmental pathway.

The AtSINAT5 E3 ligase and the AtNAC1 transcription factor participate in the auxin-dependent regulation of lateral root formation in A. thaliana, making them both attractive candidates to be recruited for nodulation. AtSINAT5 dimers specifically target AtNAC1 for ubiquitin-mediated proteasomal degradation and AtNAC1 induces transcription of genes playing a role in lateral root development (Xie et al., 2000; Xie et al., 2002). Overexpression of the SINAT5 gene and of a dominant-negative form of this gene mutated in the RING domain (SINAT5DN) results in opposite root phenotypes in A. thaliana. Both genes were ectopically expressed in M. truncatula to determine a potential nodulation function for SINAT-like genes in M. truncatula. Evidence was collected for a conserved mechanism involving SINAT functioning during lateral root development, and moreover, nodulation of the 35S:AtSINAT5DN lines showed defects during rhizobial infection and in nodule functioning which resulted in premature senescence of the nodule organ. Identification of the potential AtSINAT5 interacting M. truncatula proteins revealed 6 MtSINA proteins and a number of nodulin target proteins. Expression analysis of the corresponding genes showed that MtSINAT proteins most probably target nodulin proteins for ubiquitination and subsequent degradation at two stages of the nodulation process, in an early stage just after infection and primordium initiation and later at the point of symbiosome formation after bacterial uptake. In addition, MtSINAT proteins might contribute in triggering the nodule senescence. Hence, in this study we discovered a new class of proteins that regulate the nodulation process and might be indispensable for endosymbiosis (Chapter 5).

Three M. truncatula NAC genes which showed a high degree of amino acid similarity to AtNAC1 and that were expressed during nodulation were shown not be the functional orthologues of AtNAC1, but might have recruited a specific role in nodulation. The MtNAC1 expression during nodule development and in the infection zone of mature nodules together with a nod- or fix- phenotype upon ectopic expression and knock-down in transgenic roots suggests an important role of this transcription factor in nodulation. The RNAi lines demonstrated ectopic root formation from initiated nodule primordia, suggesting a function downstream from NF perception in setting up or maintaining hormone signaling gradients. However, further research needs to be performed to be able to position this gene in the
nodulation signaling pathways and to discover the downstream genes and will be performed with stably transformed plants (Chapter 6).

MtNAC2 and MtNAC3 genes show an upregulated expression in the roots at the onset of nodulation which is higher at the sites where nodules developed. Transgenic roots ectopically expressing or knocking down MtNAC2 showed reduced nodulation capacities, thereby pointing to a nodulation-specific function, although further study is required. A modulated expression of MtNAC3 in M. truncatula transgenic roots did not affect nodulation, suggesting that this gene has no essential role in nodulation or is redundant (Chapter 6; addendum). In summary, we provided evidence that at least one or maybe two NAC transcription factors were recruited to function in nodule development. Moreover, the RNAi phenotype of MtNAC1 showed that this gene might be linked to nodule-organ identity or at least to the set-up of gradients in a root that allow nodule formation (Chapter 6).

Hormones, such as auxin and cytokinins, play a pivotal role in nodulation. The auxin/cytokinin ratio can induce cell division and primordium formation and in addition, auxin is also present in the infection zone (Fedorova et al., 2005). In addition to hormone signaling, small peptides are believed to function in cell-to-cell communication. The CLV3/ESR-like CLE peptides are currently studied in A. thaliana and are involved in a panel of functions, such as meristem maintenance and organ development (Fletcher et al., 1999; Strabala et al., 2006), suggesting that also this gene family might be recruited for nodulation. However, the existence and knowledge on CLE genes specifically functioning in nodulation is still in its infancy. Chapter 7 presents the first investigations performed in M. truncatula to identify CLE peptides with a potential role in nodule development. Twenty MtCLE genes with very different expression patterns during M. truncatula nodulation and in a range of tissues were isolated. Moreover, the expression analysis revealed that at least five MtCLE genes might have a role during nodule development, more specifically in nodule meristem initiation and/or maintenance and in nodule senescence. A bio-assay in which ectopic expression of MtCLE genes in transgenic roots or in vitro application of CLE peptide to the roots was performed, showed the biological activity of CLE peptides in M. truncatula. These preliminary results provided evidence for active MtCLE peptide signaling in the legume M. truncatula which will be investigated in depth in the near future to gain new insides in the role of this intriguing peptide family in nodulation.
Planten vertonen specifieke kenmerken die zich ontwikkelden naargelang hun leefmilieu waardoor ze verschillen in hun reacties op de omgeving. De micro-organismen in de rhizosfeer lokken verscheidene plant responsen uit die enerzijds kunnen leiden tot een pathogene interactie met schade en ziekte voor de plant als gevolg, of anderzijds tot een symbiose die beide organismen voordeel biedt. Het overgrote merendeel van de landplanten ontwikkelde de capaciteit om een symbiotische interactie aan te gaan met fungi, wat resulteert in arbusculaire mycorrhiza in hun wortelstelsel waardoor plantengroei en ontwikkeling bevorderd wordt dankzij een verhoogde fosfaat opname uit de bodem.

Legumineuzen zijn uniek omdat deze planten species een symbiotische interactie kunnen aangaan met Rhizobium bacteriën, wat leidt tot de ontwikkeling van nieuwe organen op de wortel, de nodulen. De bacteriën opgenomen in deze nodulen reduceren atmosferische stikstof tot ammonium, wat geassimileerd wordt door de plant, resulterend in een ongelimiteerde stikstof bron voor de plant. Stikstof is een essentiële voedingsstof voor plantengroei in natuurlijke ecosystemen en agriculturele systemen en dankzij symbiotische stikstoffixatie kunnen legumineuzen aangewend worden als groenbemesting in de landbouw. Daarenboven produceren legumineuzen voedzame proteïne-rijke zaden die bijdragen tot één derde van alle stikstof nodig voor proteïnen in de menselijke voeding (Graham and Vance, 2003).

Uitgebreid moleculair onderzoek wordt wereldwijd verricht in talrijke laboratoria met als doel het symbiotische stikstoffixatie proces te ontrafelen, wat kan leiden tot diverse toepassingen die zowel op economisch als agricultureel vlak voordelen bieden. Leguminzeuzen die van belang zijn voor de landbouw, zoals soya, erwt, boon, alfalfa, enz. zijn niet optimaal voor moleculaire studies door hun grote polyploïde genomen, hun recalcitrantie, hun plant grootte en hun lange generatie tijd. In het onderzoeksveld werden daarom twee planten, Medicago truncatula en Lotus japonicus, vooropgesteld als model systemen om respectievelijk niet-gedetermineerde en gedetermineerde nodulatie te bestuderen (Cook, 1999; Handberg en Stougaard, 1992). Tevens oogt dit onderzoek op de verbetering van ‘grain legume’ opbrengsten via het verhogen van de nodulatie capaciteit en de zaadproductie. Via comparatieve genoom analyse wordt deze kennis geëxtrapoleerd naar de agricultureel belangrijke gewassen en zal mogelijks nodulatie van niet-legumineuzen bekomen worden (Hoofdstuk 1). De model planten zijn transformeerbaar wat het mogelijk maakt transgene planten met een gemoduleerde genexpressie te bestuderen. Dit terwijl menig legumineuze planten species echter recalcitrant zijn zoals bijvoorbeeld Sesbania
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rostrata. Deze tropische plant vertoont echter andere aantrekkelijke kenmerken en werd uitgebreid bestudeerd in onze onderzoeksgroep.

S. rostrata groeit in moerassige gebieden van de Sahel (Duhoux en Dreyfus, 1982) en vormt nodulen in interactie met Azorhizobium caulinodans. De omgevings situatie bepaalt de bacteriële invasieweg en het nodule type, in geaëreerde condities treedt wortel haar krulling op, terwijl ondergedompelde planten noduleren via intercellulaire ‘crack entry’ invasie aan de laterale wortelbasis en aan de adventiefwortelprimordia op de stengel (Goormachtig et al., 2004). De veelzijdige nodulatie kenmerken van S. rostrata zijn hoofdzakelijk het gevolg van een veranderde respons op ethyleen en gibberellinen (D’Haeze et al., 2003; Lievens et al., 2005; een overzicht in Hoofdstuk 2).

In Hoofdstuk 3 wordt functionele analyse van één van de genen geïsoleerd via een differentiële screening (differential display), Srchi24, beschreven. Srchi24 codeert voor een chitinase-achtig proteïne zonder hydrolytische activiteit maar met chitine bindingscapaciteit. Chitine vertoont een gelijkvormige structuur aan Nod factoren (NF), de bacteriële signaalmoleculen die het nodulatieproces in de plant aanschakelen, wat een cruciale rol voor Srchi24 suggereert in de perceptie of in de aantrekking van NFs om deze te begeleiden naar de receptor(en) in de epidermis. Srchi24 vertoont een basale expressie in de wortel en een vroege opregulatie tijdens nodulatie in de buitenste corticale cellagen en rond de infectiewiggen (Goormachtig et al., 2001). Celwandlokalisatie en chitine binding werden aangetoond voor in planta geproduceerd Srchi24, bewijs voor NF-binding kon echter niet geleverd worden. S. rostrata knock-down/out transgene wortels vertoonden ook geen verschil in nodulatie capaciteit in hydropone condities. De basale expressie van Srchi24 in de wortels en de verminderde wortelontwikkeling die werd waargenomen bij transgene wortels die een Srchi24 RNAi construct bevatten, suggereren een meer algemene rol in orgaan ontwikkeling zoals reeds voor andere chitinase-achtige proteïnen werd aangetoond (Dyachok et al., 2002) (Hoofdstuk 3).

Hoewel A. Rhizogenes transformatie van S. rostrata een waardevolle en efficiënte techniek is om transgene wortels te verkrijgen, was het niet mogelijk om transgene planten te bekomen via A. tumefaciens transformatie (Vlachova et al., 1987), wat echter wel het bestuderen van de gesynchroniseerde nodulatie op de voorbestemde adventief wortel primordia op de stengel mogelijk zou maken. Door het aanwenden van een nieuwe veelbelovende techniek, het multi-auto-transformatie vector systeem (Ebinuma et al., 1997a), werden transgene S. rostrata scheuten verkregen. Dit toont aan dat S. rostrata wel degelijk transformeerbaar is, hoewel regeneratie van het getransformeerde weefsel niet
voldoende efficiënt was om deze methode te gebruiken in verder onderzoek (Hoofdstuk 4).

Nodulen zijn, zoals lateral wortels, secundaire wortel organen (Casimiro et al., 2003; Kondorosi et al., 2005). Beide organogenesen vertonen verscheidene gelijkenissen, zoals de positionering tegenover de protoxyleempolen, de locale auxine vereiste voor initiatie, de re-activatie van de celcyclus in de pericyclus en/of de cortex, de ontwikkeling van een nieuw vasculair systeem en meristeem, en een gemeenschappelijk autoregulatorisch mechanisme die orgaan aantallen controleert (Mathesius et al., 1998; de Billy et al., 2001; Nishimura et al., 2002; Kondorosi et al., 2005). Hoogstwaarschijnlijk heeft het nodulatieproces verscheidene aspecten van het proces voor laterale wortelvorming gerecruiteerd. Lateral wortelvorming wordt uitgebreid bestudeerd in Arabidopsis thaliana, wat een nuttige basis vormt om genen te ontdekken die gerecruteerd werden uit het laterale wortelontwikkelingsproces om specifiek in nodulatie te functioneren.

Het AtSINAT5 E3 ligase en de AtNAC1 transcriptie factor maken deel uit van de auxine-afhankelijke regulatie van laterale wortelvorming in A. thaliana, wat beide gene aantrekkelijke candidaten maakt om gerecruteerd te zijn voor nodulatie. AtSINAT5 dimeren veroorzaken specifiek ubiquitine-gemediaerde proteolytische degradatie van AtNAC1, terwijl AtNAC1 de transcriptie van genen met een rol in laterale wortelvorming induceert (Xie et al., 2000; Xie et al., 2002). Overexpressie van AtSINAT5 en van een dominant-negatieve vorm van dit gen in het RING domein (SINAT5DN) resulteert in tegenovergestelde wortel fenotypes in A. thaliana. Beide genen werden ectopisch tot expressie gebracht in M. truncatula om een potenële functie in het nodulatieproces voor SINAT-achtige genen te onderzoeken in M. truncatula. Hieruit bleek dat een geconserveerd mechanisme voor SINAT-gereguleerde laterale wortelvorming aanwezig is in M. truncatula, en daarenboven werd een defectieve nodulatie verkregen in de 35S:AtSINAT5DN lijnen. Een defectieve rhizobiete infectie en nodule functionaliteit resulteerden in vroegtijdige senescentie van de nodule organen. Zes MtSINAT proteïnen en een aantal noduline substraateiwitten werden geïdentificeerd als zijnde potentiële AtSINAT5 interagerende M. truncatula proteïnen. Expressie analyse van de corresponderende genen toonde aan dat de MtSINAT proteïnen hoogstwaarschijnlijk deze nodulinen merken voor ubiquitinitatie en daaropvolgende degradatie tijdens twee stadia van het nodulatieproces, vlak na infectie en primordium initiatie en tijdens de symbiosoom vorming na bacteriële opname in de geïnfecteerde cellen. Daarenboven zouden MtSINAT proteïnen betrokken kunnen zijn in het aanschakelen van het nodule senescentie proces. In deze studie werd een nieuwe klasse proteïnen ontdekt die het
nodulatieproces reguleren en die mogelijk onmiskenbaar zijn voor endosymbiose (Hoofdstuk 5).

Voor drie *M. truncatula* NAC genen die een hoge graad van proteïne similariteit vertonen met AtNAC1 en die tot expressie komen tijdens nodulatie werd aangetoond dat deze niet de AtNAC1 orthologen zijn, maar mogelijks een nodulatie-specifieke functie recruteerden. MtNAC1 expressie tijdens nodule ontwikkeling en in de infectiezone van de mature nodulen samen met het nod- en fix-fenotype dat bekomen werd na ectopische expressie en knock-down in transgene wortels, suggereert een essentiële rol voor deze transcriptie factor in het nodulatieproces. Sommige RNAi lijnen vertoonden ectopische wortelvorming uit de geïnitieerde nodule primordia wat een rol downstream van NF perceptie suggereert, mogelijk in het opzetten of behouden van hormoon signalisatie gradiënten. Verder onderzoek is echter vereist om dit gen te kunnen positioneren in de nodulatie signaalcascade en om de downstream genen te identificeren. Dit zal uitgevoerd worden aan de hand van stabiele transgene planten (Hoofdstuk 6).

*MtNAC2* en *MtNAC3* genen vertonen een opgereguleerde expressie in de wortels tijdens de aanvang van nodulatie die sterker was op de plaatsen waar nodulen ontwikkelden. Ectopische expressie of knock-down van *MtNAC2* in transgene wortels resulteerde in een vermindere nodulatiecapaciteit wat een nodulatie-gerelateerde functie aangeeft, die verder bestudeerd zal worden. Gemoduleerde expressie van *MtNAC3* in *M. truncatula* transgene wortels beïnvloedde de nodulatiecapaciteit niet, wat impliceert dat dit gen geen essentiële rol heeft in het nodulatieproces, of dat redundantie optreedt (Hoofdstuk 6; Addendum). Samengevat kunnen we stellen dat bewijs geleverd werd voor een gerecruteerde nodulatie-functie voor minstens één, of potentiëel 2, NAC transcriptie factoren en dat MtNAC1 mogelijk bijdraagt tot de specifieke nodule-orgaan identiteit of ten minste tot de installatie van gradiënten in de wortel die vereist zijn voor een efficiënte nodulatie (Hoofdstuk 6).

Hormonen, zoals auxinen en cytokininen, spelen een fundamentele rol in nodulatie. De auxin/cytokinine verhouding kan celdeling en primordium vorming induceren, en meer nog is auxine ook aanwezig in de infectiezone van mature nodules wat op een nog onbekende rol wijst voor auxinen tijdens het nodulatieproces (Fedorova et al., 2005). Naast hormoon signalisatie zijn ook kleine peptiden van belang in cel-cel communicatie. De CLV3/ESR-achtige CLE peptiden worden momenteel bestudeerd in *A. thaliana* en zijn betrokken in diverse functies zoals in meristeem homeostase en orgaan ontwikkeling (Fletcher et al., 1999; Strabala et al., 2006) wat suggereert dat deze gen familie mogelijks
gerecruteerd werd voor nodulatie. Nochtans is slechts weinig of geen kennis verworven omtrent het bestaan van CLE genen met een specifieke functie in het nodulatieproces.

**Hoofdstuk 7** beschrijft een eerste studie die leidde tot identificatie van CLE genen met een potentiële rol in *M. truncatula* nodulatie. 20 MtCLE genen met zeer verschillende expressiepatronen tijdens *M. truncatula* nodulatie en in verscheidene weefsels werden geïsoleerd. Gebaseerd op de expressie data werden tevens 5 MtCLE genen vooropgesteld met een mogelijke nodulatie-gerelateerde functie in meristem-initiatie of -behoud en in nodule senescentie. Een bio-assay, omvattende ectopische expressie van MtCLE genen in transgene wortels of in vitro applicatie van CLE peptiden op de wortel, toonde de biologisch activiteit van CLE peptiden in *M. truncatula* aan. Deze preliminaire resultaten leverden reeds bewijs voor een actieve MtCLE peptide signalisatie in de legumineuze plant *M. truncatula* en zal verder bestudeerd worden in de nabije toekomst om nieuwe inzichten te verwerven in de rol van deze intrigerende peptide familie in nodulatie.
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Thanks to all of you!!

Dikke kus
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Biographical Information

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Born: 1 Feb 1979, Aalst, Belgium
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Marital status: unmarried

Language Skills

Dutch: native language; English: excellent; French: good; German: basic.

Education and Research Experience

October 2005 - June 2006
Ghent University/VIB, Belgium

PhD research

Project: “Functional analysis of plant genes in legume nodulation”
Coordination of the Gent laboratory contribution to WP5.2 of the European FP6 ‘Grain Legume Integrated Project’:
“Generation of the Tnt1 tagged M. truncatula mutant collection through the production of regenerated Tnt1 lines.”
Scientific advisor: Prof. Dr. Marcelle Holsters – Prof. Dr. Sofie Goormachtig

October 2001 – September 2005
Ghent University/VIB, Belgium

PhD research

Project: “Functional analysis of plant genes in legume nodulation”
Project sponsor: Predoctoral fellowship from the IWT (Vlaams instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de industrie)

Scientific advisor: Prof. Dr. Marcelle Holsters – Prof. Dr. Sofie Goormachtig

October 1997 – July 2001

Ghent University, Belgium

Master in Biochemical Sciences: graduated with great distinction


Scientific supervisor: Prof. Dr. Ann Depicker


Erpe-Mere, Belgium

High school diploma: A.S.O. Languages – Science

Additional courses and diplomas

2001 – 2002

Ghent University, Belgium

“Cell biology of plants” (Prof. Dr. Danny Geelen)
“Molecular plant-microbe interactions” (Prof. Dr. Marcelle Holsters)

2005

Ghent University, Belgium

“Academic English - Writing skills: Biomedical Sciences”
Faculty of Arts and Philosophy – Department of Language and Communication
Certificate delivered 24 June, 2005

Teaching experience


Ghent University, Belgium

Assistance in practical exercises to ‘Genetics’ course (Prof. Dr. Ann Depicker) 1st Lic. Biotechnology

2003 – 2005

Ghent University/VIB, Belgium

Supervisor of undergraduate students:
Katrien D’haeseleer, Master student Biology (Ugent): 2003 - 2004
Carmen Dorca Fornell, Socrates Master student Biology (Spain): 2004 - 2005
Magdalena Baran, Socrates student Agricultural Sciences (Poland): 2 - 7/2005

Experience abroad


ISV laboratory - Gif-sur-Yvette, Paris, France

Collaboration with research group of Prof. Dr. Eva Kondorosi during PhD research on project “Targeted protein degradation via SINA proteins controls lateral root number and is essential for nodule formation in Medicago truncatula”
Published book chapters


Peer-reviewed publications


Abstracts


Posters


VAN DE VELDE, W., DEN HERDER, G., PÉREZ, J-C., SCHROEYERS, K., CAPOEN, W., DEN HERDER, J., DE KEYSER, A., VERPLANCKE, C., GOORMACHTIG, S. and HOLSTERS M. Unraveling developmental aspects of the nodulation program in the model legume Medicago truncatula. VIB Seminar, Blankenberge (Belgium), March 16-17, 2004.

DEN HERDER, G., CAPOEN, W., DEN HERDER, J., HOLSTERS, M. and GOORMACHTIG, S. Signals involved in the establishment of a nitrogen fixing symbiosis with S. rostrata. UGent PhD symposium, ICC Gent (Belgium), May 3, 2005.


Presentations

FWO Onderzoeksgemeenschap Symposium on "Plant-Microbe Interactions", Spa (Belgium), November 10-13, 2002. Srchi24, a chitinase-like homologue without hydrolytic activity: what is the role in nodule and root development?

IUAHV meeting, Gent (Belgium), March 16, 2004. Nodule- and lateral root formation in the model legume Medicago truncatula.


IUAHV meeting, Antwerpen (Belgium), February 2, 2005. Nodulation versus lateral root development in Medicago truncatula.

Model Legume Congress, Asilomar, California (USA), June 5-9, 2005. The Sinat5/Nac1 regulatory system affects lateral root and nodule formation in Medicago truncatula.

IAPV-International Root symposium, Gent (Belgium), November 28, 2005. Root nodule initiation in Medicago truncatula.

Additional Meeting Attendances


GLIP FP6 2006 Meeting, AgroM-INRA, Montpellier (France), February 20-23, 2006.